ISOLATION, CHARACTERISATION AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF HAEMOPHILUS INFLUENZAE IN A TERTIARY CARE HOSPITAL

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University

In partial fulfillment of the regulations

For the award of the degree of

M.D. MICROBIOLOGY

Branch - IV

DEPARTMENT OF MICROBIOLOGY

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH,

PEELAMEDU, COIMBATORE, TAMILNADU, INDIA

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Certificate

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

CERTIFICATE

This is to certify that the dissertation work entitled **Isolation, characterization and antibiotic susceptibility pattern of Haemophilus influenzae in a tertiary care hospital** Submitted by **Dr. D.Sai keerthana** and this work were done by her during the period of study in this department from April 2014 to August 2015. This work was done under direct guidance of **Dr. Marina Thomas,** Professor, Department of Microbiology, PSG IMS&R.

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Acknowledgement

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Abstract

Introduction:

Haemophilus influenza is strictly a human pathogen responsible for many diseases like community acquired pneumonia, meningitis, sinusitis, epiglottitis and otitis media. Haemophilus type b is the most common strain responsible for all the infections. Non type able strains are at emerging risk. Ampicillin resistance is being reported and is due to production of betalactamases. Ampicillin resistance is being reported and is due to production of betalactamase, but rarely can also be due to aminoacid substitutions in the Penicillin binding proteins (PBP) which is known as BLNAR

AIM:

To isolate and characterize Haemophilus influenzae as pathogen from purulent respiratory samples over a period of 15 months.

Materials and Methods:

All purulent respiratory samples were processed for isolation of Haemophilus influenzae. They were identified as H. influenzae based on its X&V factor requirement and Porphyrin synthesis test. Serotyping of the isolates was done using 'b', 'a' and 'f' typing sera. Antibiotic susceptibility testing was done with reference to detection of Ampicillin resistance. Ampicillin resistance was characterized by phenotypic and genotypic methods. PCR was done for detection of TEM -1 which codes for betalactamases enzyme.

Results:

Out of 103 Haemophilus influenzae isolates, majority of them were isolated from elderly male patients (49%) and show higher incidence during the winter months .Due to the vaccination available against type b Haemophilus influenzae, Non type b Haemophilus influenzae shows higher risk (67%) when compared to type b (33%). None of the cases of type b is seen in children below 5 years of age. Haemophilus influenzae shows higher sensitivity pattern to ceftriaxone (91%),Azithromycin (96%)and Tetracycline (97%). There is no difference in the susceptibility to drugs between type b and Non type b isolates. Beta lactamase positive H. influenzae were seen in 15 isolates .Out of them, 13 showed TEM-1 beta lactamase gene by PCR.

KEYWORDS: satellitism, XV factor requirement, Porphyrin synthesis test, Fildes agar,TEM-1

Introduction

INTRODUCTION:

Haemophilus influenzae is a commensal in human respiratory tract, now considered as an important cause of community acquired pneumonia. Of the infections caused by Haemophilus influenzae, the commonly observed are pneumonia, Sinusitis, Otitis media, septicemia, meningitis, cellulitis, arthritis and epiglottitis [1]. They are related to as the typical bacterial pathogens causing community acquired pneumonia next to Streptococcus pneumoniae.

Haemophilus influenza is a short Pleomorphic Gram negative coccobacillary rod, fastidious in nature. H.influenzae was identified as Pfeiffer's bacillus in 1892 and renamed as bacillus influenza in 1918. In the 1930s, Haemophilus influenza was classified in two different categories the capsulated and the non-capsulated strains by Pittman. It is mainly recovered from the human respiratory tract and very rarely from other sites. The mode of spread of the infection is mainly airborne droplets[2] .

Among the 6 capsulated strains (a, b, c, d, e, f) type b remains the most common cause of invasive diseases [2]. Type b H.influenzae colonize the respiratory tract of children at a rate of 2- 4% which showed substantial decrease after the advent of conjugate vaccine[2].

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Nontypeable strains (Non capsulated) commonly colonize the upper respiratory tract at a rate of 30-40%. They account for 25-35% as of cause Otitis media in children less than 5 years of age and exacerbations of chronic obstructive pulmonary disease in adults.[4]

Haemophilus influenzae is confirmed by performing satellitism, in which the colony grows near the streak of staphylococcus aureus. Haemophilus influenza is a fastidious organism which requires two important factors, factor x (hemin factor) and factor v (nictoniamide adenine nucleotide factor) for its growth. [4]The most reliable method to detect the requirement of these factors is the porphyrin synthesis test identified by kilian et al.[5]

For epidemiological purposes Haemophilus influenzae can be classified in to eight biotypes based on three biochemical tests indole, Ornithine and urease. Of these biotypes, type b falls mostly in biotype I. Strains isolated from healthy nasopharynx and from respiratory sputum samples comes under biotypes II and biotypes III [6].

Among the six serotypes designated as a to f, all have antigenically distinct capsular polysaccharides. The type b strains have polyribitol phosphate capsule which is the important virulent factor. The capsulated strains can be identified by their seroagglutination with the specific antiserum. Type b alone is potentially vaccine preventable [7].

H.influenzae is sensitive to cold temperature and lyophilisation remains the ideal way for preservation.[8]

Infections caused by H.influenzae were initially being treated with cell wall active agents like Ampicillin and Chloramphenicol. Now the favored regimen is ceftriaxone, cefotaxime, tetracycline and Azithromycin. Some strains produce beta-lactamase enzyme TEM-1 which shows resistance to Ampicillin. By the year 1972 Haemophilus influenzae begin to show resistance to Ampicillin and the resistance started to spread rapidly because of production of beta lactamase enzyme .Both phenotypic and genotypic methods were used to detect Ampicillin resistance [9]

My study aim is to isolate, characterize the Haemophilus influenzae *i*n a simplified manner and also to study the drug sensitivity and resistance patterns among the isolates obtained.

Review of Literature

REVIEW OF LITERATURE:

Haemophilus influenza is known to cause infections like community acquired pneumonia, meningitis, sinusitis, acute otitis media and epiglottits. Rarely Haemophilus influenza also causes conjunctivitis and genital tract infections. Among the capsulated strains, type b is the most common cause of all these diseases. However the advent of vaccines has reduced the incidence of infections caused by type b. Non type b and other non-capsulated strains of Haemophilus are showing emerging trends [1].

Haemophilus influenza is Gram negative, Pleomorphic bacteria. In sputum samples it appears as clusters of coccobacillary forms whereas in CSF samples long, filamentous forms are seen. They are facultative anaerobic, nonmotile, nonsporing and most are capsulated. Haemophilus influenza requires two factors for its growth: factor X and factor V.

Factor X is the hemin factor and factor V is the nicotinamide adenine dinucleotide factor.Most of the species show catalase and oxidase reaction positive. Haemophlius genus consists of a total of 15 species among which Haemophilus influenza, Haemophilus aegypticus, Haemophilus haemolyticus alone requires both XV for its growth [2].

HISTORY[9, 10]:

Historical perspective of discoveries regarding the Haemophilus influenza highlights that it was Pfeiffer in 1892 identified this genus in the sputum of the patients suffering from respiratory tract infections. As time passed on, Winslow in 1917 named the species which cause these diseases such as community acquired pneumonia, meningitis, acute otitis media as Haemophilus influenza. Before the discovery of Haemophilus influenza, Koch identified a species called Haemophilus aegypticus, which caused conjunctivitis in Egypt in 1933.

Zeimann in 1967 restricted the genus Haemophilus to Gram negative rods or coccobacilli which requires factors X and V for its growth.[9]The G+C content of DNA is 37-44 mol%. After the advent of the DNA-DNA hybridization technique and 16s RNA sequence comparison, the popularity of X and V has reduced [10]. Among the different strains of Haemophilus species, based on multilocus sequence analysis Haemophilus aphrophilus and Haemophilus paraaphrophilus are classified in to Aggregatibacter_aphrophilus and Aggregatibacter paraaphrophilus.Both hemin dependent and hemin independent A.aphrophilus isolates are also identified.

Taxonomy [11]:

Haemophilus comes under the family Pasteurellaceae which also includes other genera such as Pasteurella, Actinobacillus, and Lonipinella. These genera are again grouped under the order Pasteurellales in the gamma division of the Proteobacteria.

Domain	Bacteria
Kingdom	Eubacteria
Phylum	Proteobacteria
Class	Gammobacteria
Order	Pasteurellales
Family	Pasteurellaceae
Genus	Haemophilus
Species	Influenza

SPECIES IN THE GENUS:

The genus Haemophilus consists of 15 species among which nine causes disease in humans and others in animals as per the Bergeys Manual of Determinative Bacteriology. [9]

HUMAN SPECIES	X	V	XV	ANIMAL SPECIES(V factor)
H.influenza	+	+	+	H.parasuis(swine)
H. aegypticus	+	+	+	H.paragallinarum(poultry)
H.haemolyticus	+	+	+	H.paracuniculus(rabbits)
H.parahemolyticus	-	+	-	H.haemoglobinophilus(dogs)
H.aphrophilus	+	-	-	H.felis(cats)
H.paraaphrophilus	-	+	-	
H.paraaphrophaemolyticus	-	+	-	
H.segnis	-	+	-	
H.ducreyi	+	-	-	
H. parainfluenzae	-	+	-	

Three species, namely, H.putoriorum, H.citreus, H.piscum alone do not require X and V factors. They need ATP for its growth[9]. In 1931, Pittman categorized the capsular serotypes of Haemophilus influenza as a, b, c, d, e, f out of which type b is important as it causes the invasive diseases.

In 1984, DNA-DNA hybridization has limited the studies to certain genus H.influenzae, H.aegypticus, H.segnis, H.aphrophilus, H.paraaprophilus[10]. Ribotyping studies suggest that genus Haemophilus contain the above species. In 1988, Brenner identified

aegypticus as the subgroup of H.influenzae and it causes the Brazilian purpuric fever-severe conjunctivitis in paediatric age group. But in 2002, Kilian et al disproved as there is no genetic evidence for this.

Certain studies found that H.aphrophilus, so named because of its need for carbon dioxide for its growth, is an apparent 'X factor dependent' only on primary isolation and loses it on repeated subculture. DNA-DNA hybridization techniques a show that H.aphrophilus and H.paraaphrophilus are similar but major difference is H.aphrophilus is X requiring whereas H.paraaphrophilus is V requiring [10].

In 1963 Turk, identified that the carriage rate of capsulated H.influenzae among five years of age group was around 2-4%. Other encapsulated strains 1-2% and non-capsulated strains are about 50-80%[12]. In 1964, additional species Haemophilus pleuropneumoniae was identified to cause infections such as fatal pneumonia like picture in swine. The specific feature of this species of Haemophilus along with H.parainfluenzae is that it possesses all the enzymes needed for heme synthesis and is porphyrin synthesis positive [5].

CELL MORPHOLOGY[13]:

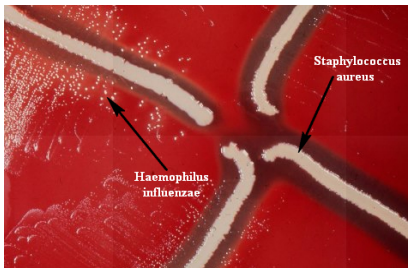
Haemophilus influenza is a small, slender, thin filamentous Pleomorphic cocco bacillary rod, irregularly stained, of size 0.3um-0.5um*0.5-1.0um with rounded ends.

Cultural Characteristics:

Good recovery of Haemophilus influenza in the laboratories depends on the proper collection, transport and use of appropriate media for isolation because of its fastidious nature. If the sample is cerebrospinal fluid, care should be taken that it is not refrigerated as the Haemophilus influenza dies off at extreme temperatures[13, 10.]

Medium used for isolation of Haemophilus influenzae:

Blood agar[10, 14]:



On blood agar they form low, translucent, convex, pinpoint colonies around the staphylococcus which provides the factor V for its growth. The colonies are bigger in size near the Staphylococcus providing V factor and as it goes away the colonies decrease in size. This is the satellite phenomenon.

Studies suggest that "Plain rabbit blood-agar" prepared by adding 5% fresh rabbit blood to sterilized Trypticase Soy Agar serves as a better media for isolation. This medium is used in microbiology laboratories for detection of Haemophilus in respiratory specimens[14]. Colonies of H.aphrophilus and H.paraaphrophilus may pit the agar leading to misidentification with Eikenella corrodens.

Chocolate agar[15]:

Chocolate agar was prepared by heating nutrient agar base with 5 per cent sheep blood at 75-80^{o} C for 15 minutes in a water bath. Studies suggest that supplementation of isovitaleX

to that of the chocolate agar enriched the growth of Haemophilus influenzae[15]. Haemophilus influenza shows a shiny, tiny colony which smells like wetfur[11].Capsulated strains show small, mucoid, translucent colonies. Chocolate agar is good in identifying the fastidious organisms such as Haemophilus, Neisseria but it cannot differentiate H.hemolyticus and parahemolyticus from H.influenzae.

A study shows the comparison of the growth of Haemophilus influenzae in enriched chocolate agar, chocolate agar with vancomycin and chocolate agar with vancomycin, clindamycin and bacitracin and suggests that the isolation of Haemophilus is about 6%, 28.5%, 59.9%. Hence chocolate agar with all the three antibiotics seems to be a better isolation medium for this pathogen from the contaminated upper respiratory tract flora[16].

Haemophilus isolation medium [17]**:**

This is a commercially available media that contains beef heart infusion, peptone, yeast extract and 5%defibrinated sheep blood containing X and V factors. Adding up of Bacitracin 300microgram/ml makes the medium more selective [11].

A study includes the comparison of different media for isolation, in which Gonococcal agar base with 1% yeast autolysate and 5% sheep blood yields a large easily recognizable colonies.

Levinthals agar [18]:

On levinthals agar colonies show iridescence under transmitted light because of grid like arrangement of the bacteria. H.paragallinarum also shows iridescence on transmitted light.

Colonies of H.parainfluenzae are smooth, wrinkled 1-2 mm in diameter. Literature studies on different media such as RPMI medium-based tissue culture media facilitate the

metabolic and genetic studies on Haemophilus influenza .The disadvantage of these media was that they did not support the growth of many non type able strains of Haemophilus influenzae[18]

Fildes agar [6, 19]:

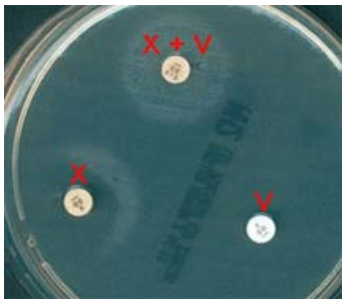
Chocolate agar or brain heart infusion agar with 5-10% fildes enrichment shows better isolation of Haemophilus. The supplement contains sodium chloride, Hydrochloric acid; defibrinated sheep blood pepsin, sodium hydroxide and chloroform. This yellow medium supports growth of Haemophilus species and other pathogens such as clostridium tetani [6].

Studies showed that Trypticase soy agar with 5% Fildes enrichment also serves as a good medium for primary isolation of Haemophilus influenzae [11].

Other media:

Five beta lactamase positive and negative strains of Haemophilus influenzae was isolated and its growth characteristics were observed in three different medias such as Mueller-Hinton broth - agar, brain heart infusion broth - agar, and tryptic soy broth - agar, and they are supplemented with (0.2% hemin-0.1% IsoVitaleX, 1% hemin-1% IsoVitaleX, 2% sheep blood, 10% Fildes enrichment, 5% horse erythrocytes, 5% Fildes enrichment, 1% supplement B, and 2% hemoglobin-1% IsoVitaleX) [19].Comparitive analysis suggests that Muller Hinton broth,brain heart infusion broth and the trypticase soy. broth supplemented with 5% fildes enrichment showed the maximum growth of more than 10^8Cfu/ml rather than 10% fildes supplement[19].

Factor X&V requirements:



Factor x:

Hemin is an enzyme in respiration. Haemophilus influenzae requires heme to supply both iron and porphyrin to the bacterium. Haemophilus influenzae also has ferrochetalase enzyme as the source for iron [20]. Since the hemolysin is not produced by the bacterium it cannot acquire its iron from the hemolysis of red blood cells.

Various sources of hemin are heme-hemopexin, heme-albumin, and hemoglobin-haptoglobin complexes [21].While growing in the anaerobic condition the requirement of X factor decreases [22]. This can be identified by supplying these factors in a Muller Hinton media. Growth can be seen around the Xfactor. Some Haemophilus strains require hemin itself since they lack ferrochetalase enzyme [20]. H.aphrophilus loses its requirement to these factors following subculture [11].

Factor v:

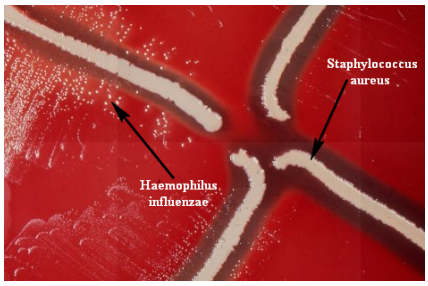
This is the second factor which is present in many tissues of plants and animals, synthesized by many bacteria except H.influenzae. It is a thermo labile vitamin, hence the name V factor [23]. In the bacterial cell growth, this factor is involved in oxidation-reduction process. Nictoniamide (NAD^+) acts as a coenzyme [24, 25.] This factor V is not readily available as it is largely intracellular. It is heat labile, destroyed at 120^0C in few minutes. Certain bacteria such as Staphylococcus aureus and some fungi produce this factor V in excess and

release them in to the medium .Haemophilus influenza utilizes these released factors for its growth.

Other dependent factors [26]:

In addition to hemin and NAD factors, certain Haemophilus species requires purine, cystiene, pantothenic acid, thiamine, uracil and cysteine for its growth. H.paragallinarum requires serum and sodium chloride, though it is not a halophilic [27].

Satellitism test[4, 10]:

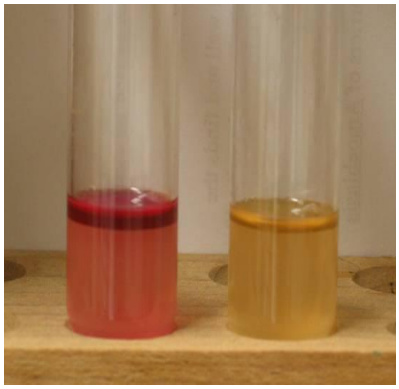


Studies suggest that H.influenzae grows as a shiny colony around the bacteria synthesizing the factor V like Staphylococci, Yeast and some fungus. The size of the colony decreases as it goes away from the factor V. This phenomenon is called satellitism and it helps in detecting these organisms in mixed cultures as well as serves as a presumptive test for genus level identification. X factor dependent Haemophilus may also grow as satellite colonies because hemin and hematin are released from lysed red blood cells by the action of hemolysin released by staphylococci.

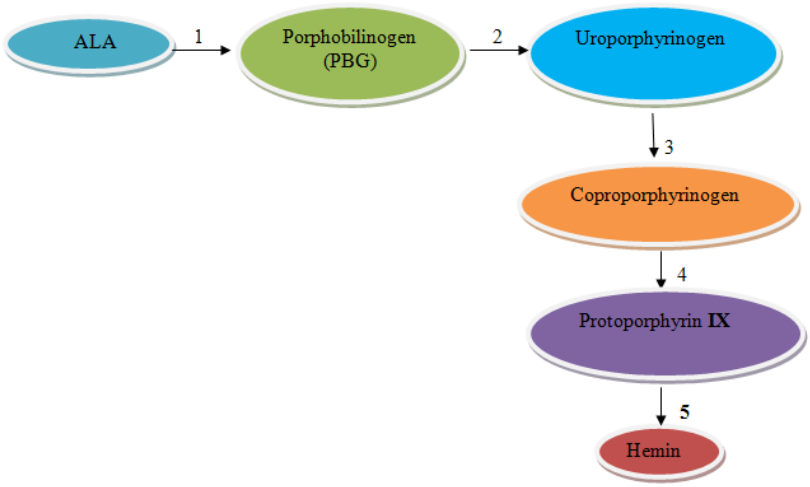
Literature studies compared five different studies such as satellitism, porphyrin synthesis test, acid production from sucrose, ONPG and indole production to differentiate Haemophilus influenzae from other species. Comparitive analysis suggest that 6% satellitism test done with impregnation of X,V on to Colombia Agar misidentified Haemophilus influenzae strains as Haemophilus parainfluenzae. Porphyrin synthesis test seems to be better in

identifying the X factor requirement. Haemophilus influenzae shows negative with acid production from sucrose while H.parainfluenzae shows positive results and this test is also comparatively good[4].

Porphyrin synthesis test [28, 29]:



The ability to synthesize porphobilinogen and porphyrins from ALA (Aminolevulinic acid) is the basis of porphyrin test, which was first described by Biberstein.



Enzymes required:

1. Porphobilinogen synthetase 2. Uroporphyrinogen I synthetase 3. Uroporphyrinogen decarboxylase 4. Coporphyrinogen oxidase 5. Ferrochetalase

Hemin-dependent strains of H.influenzae are unable to convert ALA into protoporphyrin. This test is convenient and if properly performed is the best method for demonstrating a hemin requirement. H.parainfluenzae serves as the positive control.

Literature studies by Killian suggest that the porphyrin test can be carried out using the substrate which contains 0.03352 of ALA hydrochloride,0.01972 of MgSo4.7H2O IN 100ML OF 0.1 Sorensen phosphate buffer,pH 6.9.Inoculate the organism and incubate it for 4 hours. To detect the porphyrin produced read under the woods lamp of longer wavelength. If red fluorescence is seen against white background then the organism doesn't require Xfactor or hemin for its growth [28]. The other way is after inoculation for 4 hours add kovac's reagent and then vortex well .Development of red aqueous layer indicates the lack of hemin requirement.

Literature studies compared five different studies such as satellitism, porphyrin synthesis test, acid production from sucrose, ONPG and indole production to differentiate Haemophilus influenzae from other species. Comparitive analysis suggest that 6% satellitism test done with impregnation of X,V on to Colombia Agar misidentified Haemophilus influenzae strains as Haemophilus parainfluenzae. Porphyrin synthesis test seems to be better in identifying the X factor requirement[4].

PHYSICAL REQUIREMENTS FOR GROWTH [30]:

The optimum temperature for growth of Haemophilus is 35-37 ^{o} C. They are sensitive to heat and are inactivated at 55^{o}C after a period of 30min. It grows better in aerobic than anaerobic conditions. However it grows better in the presence of 3-5% co $_{2.}$

VIRULENCE AND PATHOGENECITY:

Haemophilus influenza is mainly classified in to two major groups, namely, capsulated and non capsulated strains. Among the capsulated strains (a, b, c, d, e, f), type b is responsible for most of the infections, because of the presence of polyribosylribitol phosphate (PRP), a pentose sugar in its polysaccharide capsule.

Capsular polysaccharide:

Production of the PRP Capsule is complex involving both capsule and non capsular genetic determinants. The cap B gene of H.influenzae is a duplication of two short identical segments of17-18 Kb segments. This capB gene contains a small bex A gene that is capable of bringing out the capsule to the cell surface[13, 20]. This duplicated Cap b gene is present in 90% of H.influenzae type b whereas in the other capsulated strains this cap B gene appears as a single copy gene. Studies show that type b capsule deficient mutants can also show 50 fold increase in adherence to human epithelial cells and nearly a 300 fold increase in invasive Capillaries. The type b capsule of Haemophilus cross reacts with wide variety of bacteria including Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecium and Escherichia coli k100 [31]. Children less than 2 years do not have the anti-PRP antibodies and also Tcell indepen*d*ent nature of this age predisposes to many infections[32]

The conjugation of PRP to a protein carrier stimulates anti-PRP antibodies in young infants and this serves as basis of Hib vaccine [33].The PRP capsule also stimulates complement mediated bacteriolysis [34].

Other virulence factors [13,20]

Fimbrial adhesions:

Type b strain and 30-40% of nontypeable strains also posses the fimbrial proteins. Type b strains also posses major proteins (Hif A) and two minor proteins (Hif D,E). The minor protein Hif E is present on the tip of the fimbriae and is responsible for haemagglutination and adherence to the host cell glycoproteins and glycolipids. These are other two proteins Hif C and Hif B which is responsible for fimbrial assembly and protection of fimbrial proteins during export from the cell.

HMW1 AND HMW2 Adhesins:

These adhesin proteins are found on 70-80% of non typeable H.influenzae. Studies on non typeable H.influenzae mediated otitis media shows the presence of these HMW adhesions.

Hap Adhesin:

This is a non fimbrial adhesin protein present in all H.influenzae strains and has protease Activity. Studies show that intranasal inoculation of mice with this Hap adhesin inhibits nasopharyngeal Colonization. Other adhesion proteins are Hia, Hsf and opacity associated proteins. H.influenzae type b produce a bacteriocin called Haemocin which compete effectively with non typeable strains in nasopharyngeal colonization.

lgA protease:

These proteases inactivate Human lg A1 and account for over 90% of lgA in oropharynx. Strains of Haemophilus aegypticus and H.influenzae biogroup aegypticus are more stably piliated [35.]Piliated H.influenzae bind to erythrocytes, which contain the Anton (AnWj) antigen [36].

Lipooligosaccharide [22]:

H.influenzae has an outer membrane LPS that contains Lipid A joined via 2-keto-3 deoxy Octulosonic acid to the core polysaccharide. Since it lacks the repeating terminal side chains (O or somatic antigen) ,LPS is called as 'LOS'.

Los Lipid A is responsible for Mitogenicity, pyrogenicity in rabbits, Platelet aggregation and Lethality in mouse endotoxemia model.

LOS phase variation occurs by changes in numbers of short four nucleotide sequences resulting In the codon –anticodon reading frame.LOS of many strains H.influenzae is sialylated and helps In opsonisation and phagocytosis [37].It serves as a potential antigen for vaccination of Non type able H.influenzae .Studies suggest that conjugate vaccines with tetanus toxoid reduced the incidence of Otitis Media due to nontypeable H.influenzae in the chin chilla model [38.]

Phase variation in LOS:

The shift in the codon anticodon reading frame is due to change in the numbers of short genomic four nucleotide sequence. This result in phase variations at the genetic level [39].The sialylated LOS of H.influenzae is structurally and antigenically similar to human glycolated sphingolipids.

The complement system plays an important role in the pathogenesis of Haemophilus influenzae. LOS responds to change in the environmental conditions such as decreased oxygen level by increasing the expression of phosphorylcholine. Reason behind is H. influenzae possesses a redox-responsive regulatory system, the ArcAB two-component signaling system (TCS). Whenever the oxygen tension is low, ArcB senses the redox status of the quinone pool and autophosphorylates, leading to activation of ArcA by phosphoryl

transfer .This Arc A activates the enzymes involved in respiratory or fermentative metabolism. This Arc AB system plays an important role in the pathogenesis of nontypeable strains by controlling the expression of certain genes like lic2B and lic2C [40].

OMPs:

These are 6 to 8 OMP in the cell wall outer membrane. It is one of the useful components in vaccine.P2 and P6 are the OMPs present in which P2 occupies 50% of the OMP. Antibodies against P2 protein are bactericidal and protective in animal models [41]. By SDS – PAGE, nine OMP subtypes have been identified. These subtypes accounts for majority of infections worldwide. The epitopes of P2 is immunogenic and are directed against the non type able H.influenzae infections[42].There is a variation in the expression of amino acid sequence in surface epitopes of P2 but those which is embedded inside the outer membrane are conserved[43]. P6 protein is about 1-5% on the surface of both type able and nontypeable strains[44.]Antibodies against P6 are bactericidal in the infant rat model and in the chinchilla model of otitis media [45].There is development of CMI and mucosal IgA response in the infant rat by the intranasal immunization of purified P6 vaccine[46]. P6 is highly conserved at the genetic level and warrants as a potential candidate in vaccine against nontypeable H.influenzae [47].

CLASSIFICATION[9], [11]:

Haemophilus species identification can be done based on the acid and gas production from carbohydrates. Haemophilus influenzae shows catalase and oxidase test positive, nitrate reduced to nitrite and most strains of Haemophilus influenzae is urease positive which helps in biotype identification along with indole and ornithine.

Species	catalase	oxidase	indole	urease
H.influenzae	+	+	D	D
H.hemolyticus	+	+	+/-	+
H.haemoglobinophilus	+	+	+	-
H.ducreyi	-	+	-	-
H. aphrophilus	-	-	-	-
H.parainfluenzae	+/-	+	D	D
H.parahaemolyticus	+/-	+	-	+

Based on acid production:

Species	Glucose	fructose	galactose	lactose	mannose	sucrose	trehalose	xylose
H.influenzae	+	-	+	-	-	-	-	+
H.hemolyticus	+	W	+	-	-	-	-	V
H.haemoglobinophilus	+	W	+	-	-	-	-	V
H.ducreyi	-	-	-	-	-	-	-	-
H.aphrophilus	+	+	+	+	-	+	+	-
H.parainfluenzae	+	+	+	-	-	+	-	-
H.parahaemolyticus	+	+	+/-	-	-	+	-	-

INFECTIONS[12]:

Capsulated strains of about 2-4% cause infection in young children and infection is commonly bacteremic resulting in Meningitis, epiglottitis, pneumonia, suppurative arthritis, osteitis, otitis media, cellulitis and pericarditis.

Non-capsulated strains of about 50-80% cause exacerbations of chronic bronchitis, otitis media, and conjunctivitis and paranasal sinusitis in adults and it is rarely bacteraemic.

Meningitis:

Meningitis is less common between two months of life to 4 years. In Britain, along with Neisseria, type b is mostly responsible for meningitis and accounts for 1 in 500 lives in the first 5 years of age [48]. Even in USA, the infection rate is 1 in 500. Among Alaskan Eskimos the population risk is 1 in 50 but it is not due to special virulence of the type b strains or to any special susceptibility of Eskimo children [49]. It is because of greater exposure of the children to the bacteria before they developed immunity.

Clinically H.influenzae type b meningitis is similar to meningococcal meningitis. It is usually preceeded by viral upper respiratory tract infection and otitis media. The symptoms include fever, malaise and vomiting. Nuchal rigidity is usually absent. Complications include brain abscess [50].

Pneumonia [10, 12]:

Pneumonia is usually lobar or segmental and the picture is similar to pneumonia caused by pneumococcal and even in its radiological appearance. Most commonly this bacterium is identified from respiratory samples. Clinical manifestations include cough, sputum, and pleuritic pain. Bacteraemic pneumonia is seen in elderly patients.

Otitis media[10]:

About one third of Nontypeable strains of Haemophilus commonly cause otitis media. It is associated with pain and fullness in ears, usually bilateral, bulging tympanic membrane. Swabs from the drainage of ear canal can be taken for cultures, otherwise aspiration should be done.

Bacteraemia [51, 52]:

Bacteraemic infections caused by H.influenzae are usually not either localized or severe enough to hospitalize. Four cases had been identified in which trauma led to bacteraemic infection. They were

1. Child crushed her hand in the door developed type b suppurative arthritis.
2. Foreign body aspirated and its removal led to type b epiglottitis
3. H/O fall led to type b meningitis
4. Chronic bronchitis

Animal models were used to demonstrate the spread of bacteraemic infections of type b by using infant rat models. The bacteria was introduced through the nasal mucosa and Haemophilus influenzae starts spreading from the nasal mucosa ,enters the circulation and finally led to meningitis rather than causing meningitis through cribriform plate[12].

OTHER INFECTIONS[12]:

Conjunctivitis:

It was Koch in 1883 who first described an organism which is classified under the genus Haemophilus. It is called as Koch-weeks bacillus or Haemophilus aegypticus. Still there is confusion whether it is a separate species or a biogroup of H.influenzae [53]. In Britain, these conjunctivitis cases occur as sporadic cases and are mainly due to non capsulated strains [54].

Genital tract infections:

Colonization of healthy vagina with non capsulated Haemophilus is very rare. However it causes salpingitis and tubo-ovarian abscess. Infection is more common among

those who have intrauterine devices or any previous infection [55]. Perinatal infections may occur and the presenting features include turbid foul-smelling amniotic fluid.

Haemophilus influenzae can also be found rarely in faeces [56]. It may also be associated with the presence of calculi or any abnormality of the urinary tract [57] H.parainfluenzae and H.aphrophilus are more involved in endocarditis than H.influenzae.

ANIMAL PATHOGENICITY:

An early study on animal pathogenecity suggests that the type b capsulated forms are highly pathogenic on injecting into rabbits[58]. The same result has been derived in studies conducted on mice [59] and chick embryos [60].

The situation was complicated in 1960 that other capsulated strains of types can also be virulent for mice and showed that 2 strains, one derived from type b and other from type d strain. Type d strain was highly virulent in mice. From this it was deduced that virulence depends on the somatic as well as capsular composition [61].

In 1981, several studies were conducted on 3 months old infant rat to study the pathogenecity of influenzae.

The main findings are [62]:

- On IV inoculation of 10^5 organisms, other than type a or b, all other types disappeared rapidly. Type 'a' then slowly disappeared leaving behind type b.
- On peritoneal inoculation even in low doses only the type b strain caused bacteraemia.
- On intranasal inoculation, type b and type d capsulated strain of the same and the non capsulated strain were equally efficient in establishing colonization of nasopharynx but type b caused bacteraemia and meningitis.

In 1981, experiments on infant rat of same breed suggest that both type b & type d strains can cause bacteraemia on intranasal inoculation but type b strains are more virulent when given subcutaneously. The difference arises because of variation in bacterial strains and the inoculation technique [63].

The final conclusion from the rat experiments suggests that capsulated Strains of Haemophilus influenza; particularly type b is mostly responsible for all pathogenic infections. The type b capsule has a special virulent factor PRP (Polyribitolribose phosphate, a pentose sugar) which is the major pathogen.

Non-typeable strains of Haemophilus influenzae [64]:

These strains are seen in the nasopharynx of the children and cause both localized and invasive infections also. Because of the advent of the type b vaccination, these nontypeable strains have taken over importance as it causes bacteraemia, meningitis and respiratory tract diseases.

Both the typeable and nontypeable strains originate from the common noncapsulated ancestor. The nontypeable strains carry number of adhesive factors like HMW1/HMW2 proteins, Hia, Hap and pili that play a major role in virulence.

EPIDEMIOLOGY[6, 29]:

Haemophilus constitute 10% of the normal bacterial flora of the upper respiratory tract. The species H.parainfluenzae accounts for three-fourth of the oral cavity but is absent in nasal cavity. Non capsulated strains of H.influenzae constitute less than 2% of the total bacterial flora in the pharynx and the nasal cavity of the healthy children.

As age increases, colonization of H.influenzae decreases but in contrast, patients with chronic obstructive disease often persistently colonize single or multiple clones of non capsulated H.influenzae.

H.influenzae infections often show seasonal variations and it is found to be most common in winter months .Crowding is one of the important factor in the transmission of the disease. The advent of vaccines especially against type b had shown dramatic decrease in the infection rate.

MODE OF TRANSMISSION[6, 29]:

Haemophilus influenzae infections spread mainly as droplet infection. The portal of entry is usually through nasopharynx. The incubation period is 2-4 days. This infection is not highly contagious. Chances of secondary infections are more common among close contacts. Once the antibiotics are started, the chances of spread are very minimal.

BIOTYPING [6]:

For epidemiological purpose the Haemophilus species are grouped under eight different biotypes based on three tests Indole, ornithine and urease.

TEST	I	II	III	IV	V	VI	VII	VIII
INDOLE	+	+	-	-	+	-	+	-
ORNITHINE	+	-	-	+	+	+	-	-
UREASE	+	+	+	+	-	-	-	-

It was in 1976, Kilian rekindled interest in the biochemical characterization of H.influenzae. He classified biotype 6 in which the species showed positive reaction to ornithine alone. A carriage study was carried out on the infants in Asaro valley of Eastern Highlands of Papua, New Guinea and a new H.influenzae strain 03635 was identified and all

three biochemical tests was done and it showed positivity to indole alone. This doesn't fit in to any of the six biotypes as described by Kilian and so new biotype VII was designated to this strain 03635 [65].

The rapid kits are available for the detection of the biotypes such as IDS RapID NH system, the NHI card, and the API NH strip and in the study by kilian, the API NH strip seems to be better than others in identifying the biotyoes.If there is much discrepancy in comparison of these three kits we go in for indole, ornithine, urease test[66.] The IDS RapID NH system was inferior to both of the other test systems because of false-positive ornithine decarboxylase results.

SEROTYPING:

Due to difference in the pathogenecity caused by capsulated or non capsulated strains, the need for detecting the type able strains gained importance. Of that, type b is the most common. Advent of vaccines against type b Haemophilus influenza had let down the infections caused by them. Studies show that still there is a rise in infections caused by Haemophilus influenzae is because of other capsulated strains and the nontypeable strains. A study on Alaskan residents of age group around 10 years and above show a dramatic increase in infections from 0.5 to 1.1 per 1,00,000 years [68].

Among the serotypes Haemophilus influenza type f has gained importance now. The incidence of type f has increased from 0.5 cases per 1,000,000 populations on 1989 to 1.9 cases per 1,000,000 populations in the year 1994.Out of that 72% of adults and 26% of children less than 5 years are commonly affected and the diseases caused are mainly the respiratory tract infections[69].

Like Haemophilus influenza type f, type a also gained importance. Study done in Salvador, Brazil says that after the advent of Hib vaccination the meningitis because of type b has decreased to 69% whereas the meningitis due to type a has increased 8-fold. Pulse field gel electrophoresis shows that type a belonged to 2 clonally related groups that were present before Hib vaccine emerged. Therefore, Hib immunization led to increased risk for H. influenzae type meningitis through selection of circulating H. influenzae type a clone. So monitoring of vaccination becomes essential[70].

Recent study on Haemophilus influenza in a hospital in Spain, showed that a 5 month old non immunized infant with history of fever and lethargy and her lumbar puncture culture showed the presence of mucoid colonies of Haemophilus influenza and the isolate was sent to the national reference laboratory at the Istituto Superiore di Sanita` (Rome), where serotyping by slide agglutination and capsular genotyping by PCR were performed and was identified as type e of Haemophilus influenza. Similar picture was identified in 26 adults also. Haemophilus type e clinical scenario is not much similar to type b and it occurs as opportunistic pathogen [71.]

Study on the epidemiology of serotype a in north American countries shows that among 88 typeable isolates, 42 (48%) were *H. influenzae* type a and it mainly affects the young children. Common clinical manifestations included meningitis, pneumonia, and septic arthritis. Overall annual incidence was 0.9 cases per 100,000 populations [72].

OTHER TYPING METHODS[67]:

- Sub typing based on OMP proteins, LPS or isoenzymes.
- DNA based techniques like DNA fingerprinting, Ribotyping and Pulsefield gel electrophoresis.

- Multilocussequence typing-Best method where the seven housekeeping genes (adk, atp G, frd B, fuc K ,mdh, pgi, rec A. The advantage of this method is that it is electronically portable and easily comparable with other laboratories.

ANTIBIOTIC SENSITIVITY PATTERN:

Most of the strains of Haemophilus influenzae respond to Ampicillin, Cephalosporins, Chloramphenicol, Sulfonamides, Tetracycline and the Macrolide antimicrobials. Ampicillin is used by most of the clinicians as the empirical treatment on suspicion of Haemophilus influenzae[68].

By the year 1972 Haemophilus influenzae begin to show resistance to Ampicillin and the resistance started to spread rapidly because of production of beta lactamase enzyme[75, 76]. This beta lactamase enzyme was found to be very similar to R factor carrying type IIIa beta lactamase of E.coli and was named as TEM-1[77].The resistance spreads through two types of plasmids 30-megadalton and 3-megadalton,out of which 3-megadalton is common among many different species[78,79].

Another beta lactamase enzyme is reported which is also plasmid mediated and cannot be detected by chromogenic cephalosporin method unlike TEM-1 [80]. Because of the plasmid mediated beta lactamase enzyme production many strains show resistance to Ampicillin.

Other methods of resistance are due to alteration in the target penicillin binding proteins. Literature studies says that Ampicillin resistance among Haemophilus influenzae is because of TEM-1 gene which is present in 90-95% of strains and 5-10% percent shows the presence of ROB-1 gene[68].Another enzyme called VAT-1 gene can also lead to beta lactamase production but it is very rare[11].

One of the simple method to detect ampicillin resistance in laboratories is the usage of Haemophilus test medium (HTM),Muller Hinton-chocolate agar and chocolate agar with 1% Fildes enrichment.HTM includes Mueller-Hinton medium with 15 micrograms of hematin per ml, 15 micrograms of NAD per ml, and 5 mg of yeast extract per ml as growth-promoting additives. A better result is seen on using Haemophilus test medium when compared to other Media [81].

DETECTION OF BETA LACTAMASE ENZYME:

1. Phenotypic method
2. Genotypic method.

Phenotypic methods:

- Acidimetric method[73]:

In this method we test the hydrolysis of the beta lactam ring and the production of penicilloic acid which is indicated as a colour change. To 1,000,000 units of benzylpenicillin-5% of 0.5ml phenol red solution diluted in 4.5ml distilled water .Add a drop of 1M NaOH. Inoculate the bacteria. Colour change from violet colour to yellow colour within 1 minute indicates beta lactamase activity.

- Iodometric method [74]:

The concept of this iodometric method is that the penicillin molecule does not bind to iodine directly where as if the beta lactamase activity is present, the penicillin gets reduced to penicilloic acid and this product is capable of binding to the iodine .so there is no chance for further reaction with starch and the end product is colorless.

- Chromogenic cephalosporin method [73]:

The beta lactamase activity can be detected by change in colour from yellow to red in one min by using nitrocefin dissolved in DMSO.

These three methods rapidly detect beta-lactamase activity in Haemophilus influenzae. The chromogenic cephalosporin method was easy to perform and the reagents could be stored for up to three weeks. The phenol red method was simple to perform but the iodometric method was more time consuming. All three tests gave identical results [73].

Genotypic method:

Polymerase chain reaction is used to determine the TEM-1 mediated resistance. Primers specific for bla TEM and blaROB ampicillin resistance genes were selected and their specificity is checked using series of E.coli isolates with ampicillin resistant genes[82].

Literature studies suggest that beta lactamase activity is positive in 15% overall.TEM-1 mediated resistance is 93.7% and ROB-1 is 4.6% respectively.ROB-1 genes are more commonly isolated from people of North America and is more sensitive to cefaclor and cefprozil than TEM-1 beta lactamases[83].

PRESERVATION AND STORAGE[8, 11]:

Haemophilus influenzae strains can be stored in chocolate agar slopes for about 4-6 weeks. If the Trypticase soy broth or the Brain heart infusion broth supplemented with glycerol is used, recovery of Haemophilus is good .Supplementation of the skimmed milk with glucose, yeast extract and glycerol maintain the viability of Haemophilus influenzae at a highest rate both at -20^{o}C AND -70^{O}C.High survival percentage can be attained when thawing at room temperature does not exceed more than 3 hours. Viable cell counts were carried out after 0, 10, 30 and 60 days and 4, 6 and 12 months of storage to determine viability. Lyophilized strains can be stored for several years.

Hib VACCINES:

Vaccines are prepared against type b polyribosylribitiol phosphate capsule as it is an important virulence factor. Response to this vaccine by infants less than 2 months is very poor. So a combination of four vaccines (PRP-diptheria toxoid conjugated vaccines (PRP-D), Outer membrane protein of Neisseria meningitides conjugated (PRP-OMP), Cross reacting mutant diphtheria protein (PRP-HbOC), Tetanus–toxoid conjugated vaccine (PRP-T) [84] was tried and they showed good results. Concept behind these conjugated vaccines is to increase their immunogenicity.

Epidemiology and clinical manifestations of Haemophilus influenzae infections have undergone dramatic changes in the last 2 decades[84]. The use of Haemophilus influenzae type b vaccines has eradicated Hib disease more widely. These vaccines induce humoral responses and also reduce the nasopharyngeal carriage of Hib. The vaccines had a great impact in reducing the incidence of the Hib disease[**85**].

Studies suggest that pneumococcal vaccines also have a great impact over the prevention of Haemophilus influenzae[85]. The incidence of Haemophilus influenzae type b meningitis (median-21 cases per 1,00,000 population per year; range-1 to 95 cases per 1,00,000 population per year) in Asia and central European countries is less than other areas where the median is 5 and 11 cases per 1,00,000 per year respectively [86] was also noted.

Aims & Objectives

AIMS:

To isolate and characterize Haemophilus influenzae as pathogen from purulent respiratory samples over a period of 15 months.

OBJECTIVES:

- To isolate and characterize the Haemophilus influenza from the purulent respiratory samples suspected of Community acquired pneumonia.
- To identify biotypes and serotypes of Haemophilus influenza.
- To detect and characterize Ampicillin resistance in Haemophilus influenza by phenotypic and genotypic methods

Materials & Methods

Materials and Methods

Study area: PSG Hospitals, Coimbatore

Study type: Prospective, Observational study

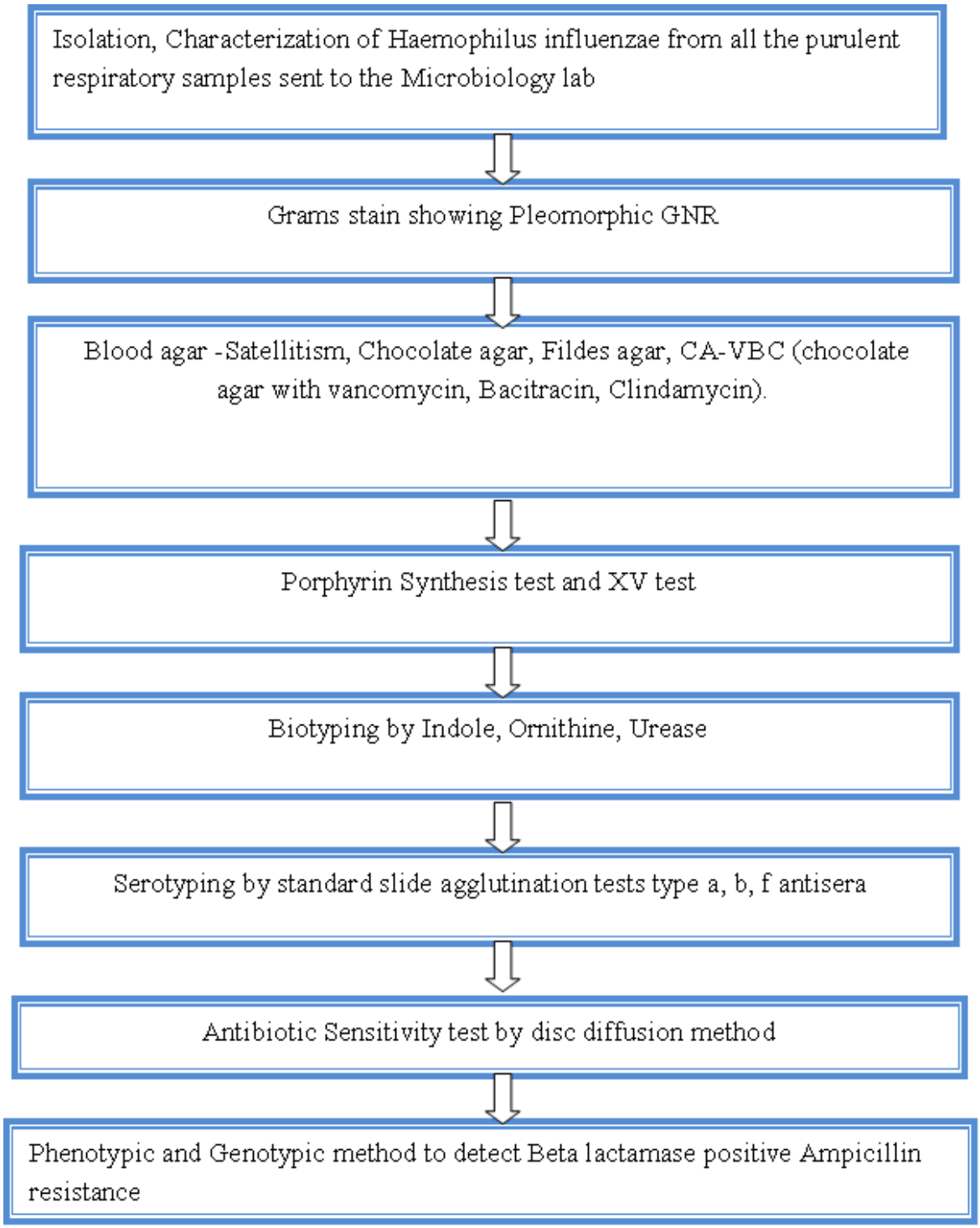
Study population: All purulent respiratory samples from patients suspected of having pneumonia/respiratory tract infections will be subjected to Gram stain and those showing Bartlett's scoring of pus cells 10-25/LPF and pus cells ≥ 25/LPF were included in the study.

Study period: 15 months

ETHICAL CONSIDERATION:

The study was undertaken in the Diagnostic Laboratory, Department of Microbiology, PSG Hospitals. Institutional Human ethical clearance was obtained. Sputum samples were collected from inpatients and outpatients with symptoms of respiratory infections.

Methodology:



Isolation of Haemophilus influenzae:

All samples as indicated above were inoculated on Chocolate agar, Fildes agar, Chocolate agar with antibiotics (Vancomycin, Bacitracin and Clindamycin), and Blood agar with Staphylococcus streak. (Composition of plating media in appendix)

Blood agar [14]:

Blood agar was streaked with the sample along with Staphylococcus aureus and it was incubated at 37^0 C in the presence of 5-10% co_2 in a candle extinction jar for 24 hours. They form low, translucent, convex, pinpoint colonies around the staphylococcus which provides the factor V for its growth. The size of the colony decreases as it goes away from the staphylococcus streak. This is the satellite phenomenon shown by Haemophilus influenza.

Chocolate agar[11]:

Heated blood agar or the Chocolate agar which provides both the hemin and NAD factor was streaked with the sample and it was incubated at 37^0 C in the presence of 5-10% co_2 in a candle extinction jar for 24 hours. Haemophilus influenza shows a shiny, tiny, smooth, flat colony which smells like wetfur[11].

Fildes agar [6:]

Fildes enrichment is an enzymatic digest of blood used to enhance the growth of fastidious organisms. The resultant digest is rich in growth factors including X and V factor required by Haemophilus influenzae. To the BHI chocolate agar of 100 ml, add 5ml of Fildes enrichment broth and allow the media to set. The sample is streaked and incubated at 37^0C in the presence of 5-10% co_2 in a candle extinction jar for 24 hours. Colony morphology is very similar to that on chocolate agar.

Chocolate agar with VBC [16]:

The CHOC-VBC media was made by preparing chocolate agar and the following antibiotics were added in concentrations of 5 microgram/ml Vancomycin, 300 microgram/ml of Bacitracin, 1 microgram/ml of clindamycin [16].After that the media is allowed to dry and the samples are directly streaked on to the plates and incubated at 37^0C in the presence of 5-10% co_2 in a candle extinction jar for 24 hours. Haemophilus influenza shows a shiny, tiny, smooth, flat, slightly yellow colony which smells like wetfur[11]

Identification of Hemophilus influenza:

Suspect colonies were those colonies which were non hemolytic, smooth, flat or low convex, shiny, easily emulsifiable and smelling like 'wet fur' [11] 'pungent odour' [10].

All suspect colonies were subjected to Gram stain[6] and Satellitism for initial identification. Gram stain characteristically shows Gram negative, small pleomorphic bacilli. The isolate was confirmed as Haemophilus influenzae by a negative porphyrin test and its requirement of X and V factor.

Porphyrin synthesis test [6]:

This test indicates the absence of requirement of X factor. If &Amino levulinic acid is provided to the bacterium, it synthesizes it and excretes porphobilinogen and other porphyrins. This test is carried out by using kovac's indole reagent (Para dimethyl amino benzaldehyde) and formation of pinkish red colour in the lower water phase indicates the synthesis of porphyrin and absence of X factor requirement.

X and V requirement [10]:

Make a lawn culture in the Muller Hinton agar plate and keep the commercially available discs X and V from HIMEDIA laboratories. The X factor (hemin) and V factor (Coenzyme-

Nicotinamide adenine dinucleotide NAD+) are impregnated on the sterile filter paper discs of diameter 6 mm. If both X and V factors are required, then the organism will grow only in the vicinity of the X+V factor discs.

Typing:

1. Biotyping was done using the biochemical tests as follows Indole production, Ornithine decarboxylation and urease production [6]
2. Serotyping was done using –Becton-Dickinson --- Kit as follows.

Biotyping:

TEST	I	II	III	IV	V	VI	VII	VIII
INDOLE	+	+	-	-	+	-	+	-
ORNITHINE	+	-	-	+	+	+	-	-
UREASE	+	+	+	+	-	-	-	-

Indole production [6]:

Indole production by Haemophilus influenza is detected by adding a loopful of bacteria to the peptone. Incubate for 24 hours at 37^{o}C.Next day add Kovac's reagent 5 drops.

Appearance of pink colour on the alcohol layer indicates indole production.

Ornithine decarboxylation[6]:

Inoculate light inoculum in to the medium and incubate at 37 ^{o} c for 18-24 hours. A control tube without an amino acid should be inoculated .Overlay the test and the control tubes with sterile paraffin oil .Under these conditions the oxygen in the medium is used up by the organism and this will control the pH.

Purple color change in the medium indicates the decarboxylation of ornithine.

Urease production[6]

To determine the ability of the organism to split urea, and two molecules of ammonia is formed by the action of the constitutive enzyme urease with resulting alkalinity. Inoculate heavy inoculum in to the medium and incubate at 37 ^{o} c for 18-24 hours. If the organism utilizes the urea, ammonia is formed

Appearance of pink colour indicates positive reaction.

Serotyping:

As per the manufacturer's kit (BD Difco catalogue no. Type b-222361

Type a-222501

Type f-227921)

Principle:

Serological confirmation involves the reaction in which the microorganism (antigen) reacts with its corresponding antibody. This in vitro reaction produces macroscopic clumping called agglutination. The test is done from the 24 hours growth of the isolate in chocolate agar. Transfer a loopful of growth to a drop of 0.85% saline on a clean slide. Emulsify in it. Rotate the slide for one minute. Observe for auto agglutination. Auto agglutination if present, the culture cannot be tested. If auto agglutination is not seen then we can proceed with further steps.

Culture isolates are tested with Difco Haemophilus influenzae monospecific antiserum b, a and f.

Steps in the procedure:

1. Put 1 drop of the Difco Haemophilus influenzae antiserum on a clean glass slide.
2. Transfer a loopful of growth to the drop of antiserum and mix thoroughly.

3. Rotate the slide for 1 min and read for agglutination of 3+ or more agglutination within 1 min.

Antibiotic susceptibility testing:

Antibiotic susceptibility testing was done by Kirby Bauer disc diffusion method on Chocolate agar [30].

In 100 mm Petri dish plates, 6 discs were kept at a distance not more than 24 mm apart. The isolates were tested for its susceptibility to Ampicillin, Ceftrioxzone, Azithromycin, Tetracycline, Ciprofloxacin, Cotrimoxazole, and Rifampicin. Make a lawn culture on the chocolate agar using direct colony suspension and incubate at 37 ^{0}c for 24 hours [87]. Next day the zone of inhibition was measured by holding the petri plate a few inches above a black background illuminated with transmitted light.

As per CLSI guidelines 2014 the sensitivity zones were noted down which is shown in the following table.

DRUG	SENSITIVE ZONE (>)	RESISTANT ZONE
Ampicillin	22mm	18mm
Ceftrioxzone	26mm	-
Ciprofloxacin	21mm	-
Azithromycin	12mm	-
Tetracycline	29mm	25mm
Cotrimoxazole	16mm	10mm
Rifampicin	20mm	16mm

Detection of Ampicillin resistance

1. Phenotypic method
2. Genotypic method

Phenotypic method:

- Acidimetric method [88]:

2 mL of 0.5% aqueous phenol red solution was diluted with 16.6 mL distilled water and 1.2 g of benzyl penicillin was added. The pH was adjusted to 8.5 with 1 M NaOH. The resulting solution was violet in colour, and stored at –20°C. Before use, 100 µl portions were distributed into tubes or microtitre wells and inoculated with bacteria from culture plates (not broth) to produce dense suspensions. A yellow colour within 5 min indicates β-lactamase activity. Positive and negative controls must be run in parallel. This test can be carried out in filter paper strip method also.

Genotypic method:

Molecular method PCR

Principle:

Polymerase chain reaction amplifies a specific target region of the template DNA strand. Using suitable primers and cycling conditions the bla_{TEM-1} gene was amplified and identified.

Steps involved:

- DNA extraction.
- Amplification using Thermo cyclers
- Agarrose gel electrophoresis

DNA extraction:

- Colonies of Haemophilus influenza is emulsified in to BHI broth and allowed to grow overnight.
- The turbid broth is centrifuged at 5000 rpm for fifteen minutes.
- The pellet is then transferred to small eppendorph tubes and 0.5 ml of distilled water is added to pellet.
- Vortex and then centrifuge at 10,000 rpm for 5 minutes.
- The DNA extraction is done by boiling method .Keep the above mixture in water bath at 95 ^{0}C for 20- 30 minutes.
- The tubes were cooled to room temperature, centrifuged at 3000 rpm for five minutes.
- The supernatant was used as the DNA extract.

Primers used for Amplification: [82]

Gene	Primers	primer sequence	Base pair
bla $_{TEM}$ -1	Forward	5'TGG GTG CAC GAG TGG GTT AC 3'	526
	Reverse	5' TTA TCC GCC TCC ATC CAG TC 3'	

For one reaction to take place 12.5 micro liters of the master mix , 5.5 micro liters of PCR water and 2 micro liters of the forward and reverse primers are mixed up. From that 20 micro liters is added up to the 5 micro liters of the DNA extract and then amplified using a thermo cycler.

Amplification using thermo cycler [82:]

The bla$_{TEM}$ assays used the following cycle parameters in the thermo cycler:

- 94 ^{0} C for 5 min
- 30 cycles of 94 ^{0} C for 2min
- 57 ^{0} C for 1 min.
- 72 ^{0} C for 2 min followed by
- 72 ^{0} C for 10 min.
- 4 ^{0} C for 5 min.

Gel preparation:

preparation of working buffer using millipore water:

- Tris base ……..10.78 gm
- Boric acid…….5.50 gm
- EDTA……3.72 gm
- Distilled water……1000ml

pH 8.3

- Working buffer…….1X

(10 times dilution of 10X is 1X-450 ml of Millipore water (nuclease free water) +50ml of 10X buffer)

- Ethidium bromide ……..10mg/ml

Gel preparation:

To 40 ml of 1X buffer, 0.35 gm of Agarose was added .It was mixed well and kept in microwave oven for 2min.After cooling for few minutes 1microlitre of ethidium bromide was added. Finally the mixture was poured off in to the trough with the comb and allowed to set for ten minutes.

Five microlitres of each amplicon was loaded with 3 microlitres of methylene blue dye. Test samples, positive and negative controls were loaded into appropriate wells.100 basepair plus DNA ladder, ready to use was used as the molecular ladder (Fermentas, Genetix Biotech Asia PVT Ltd, Bangalore).

Then the gel was transferred to the tank with the current passing from cathode to anode at 55 Volts for ten minutes and then the voltage is increased to 99 V for 45 minutes.Gel documentation was done under UV light using GELDOC XR.

Results and interpretation:

The isolate was considered positive for bla $_{TEM-1}$ gene when a band was obtained at the level of the positive control band (526 bp), which was comparable to the 526 bp of the molecular ladder. Isolates which did not possess the gene did not produce any band.

Results & Analysis

Results and analysis:

During the study period from April 2014 to August 2015, a total of 4296 respiratory samples from patients suspected to have pneumonia were received in our hospital. Out of these in 2756 samples the pus cells per LPF was less than 10, therefore these samples were considered as inappropriate for culture and none yielded growth of Haemophilus influenzae.

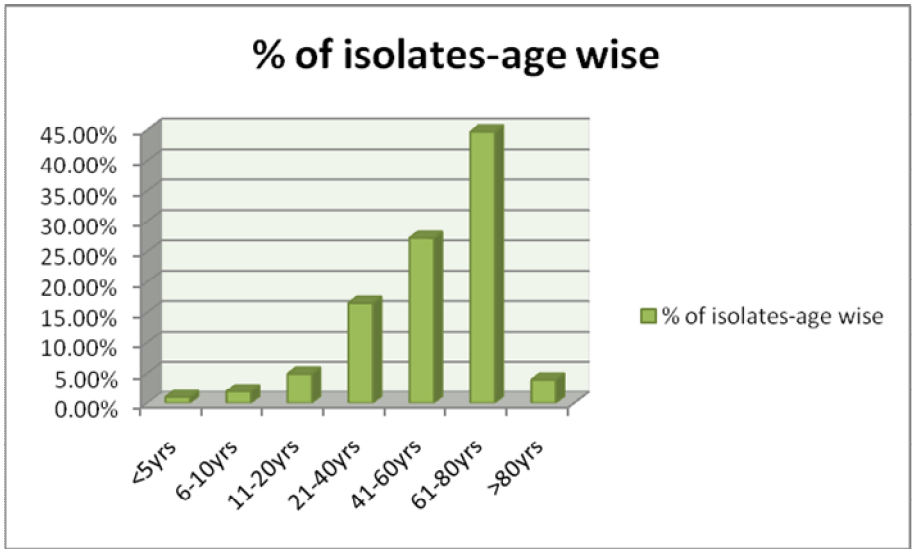
Cultivation of the remaining 1540 samples (968 samples were with pus cells > 25/LPF and 572 samples were with pus cells 10-25/LPF), yielded Haemophilus influenzae in 103 cases. Among the 103 isolates, 79(76.6%) were from sputum samples with pus cells > 25/LPF and 24(23.3%) were from sputum samples with pus cells 10- 25/LPF.

All the Haemophilus influenzae isolates were further analyzed by Biotyping and Serotyping methods. The study includes patients of all age groups.

Age wise distribution of Haemophilus influenzae:

Among the Haemophilus influenzae isolates, 44.7% of them seen in age group 61-80 years, 27.2% from 41-60 years, 16.5% from 21-40 years, 4.8% from 11-20 years, 1.94% from 6-10 years and 0.9% from < 5 years and 3.9% from >80 years of age. (Illustration No. 1)

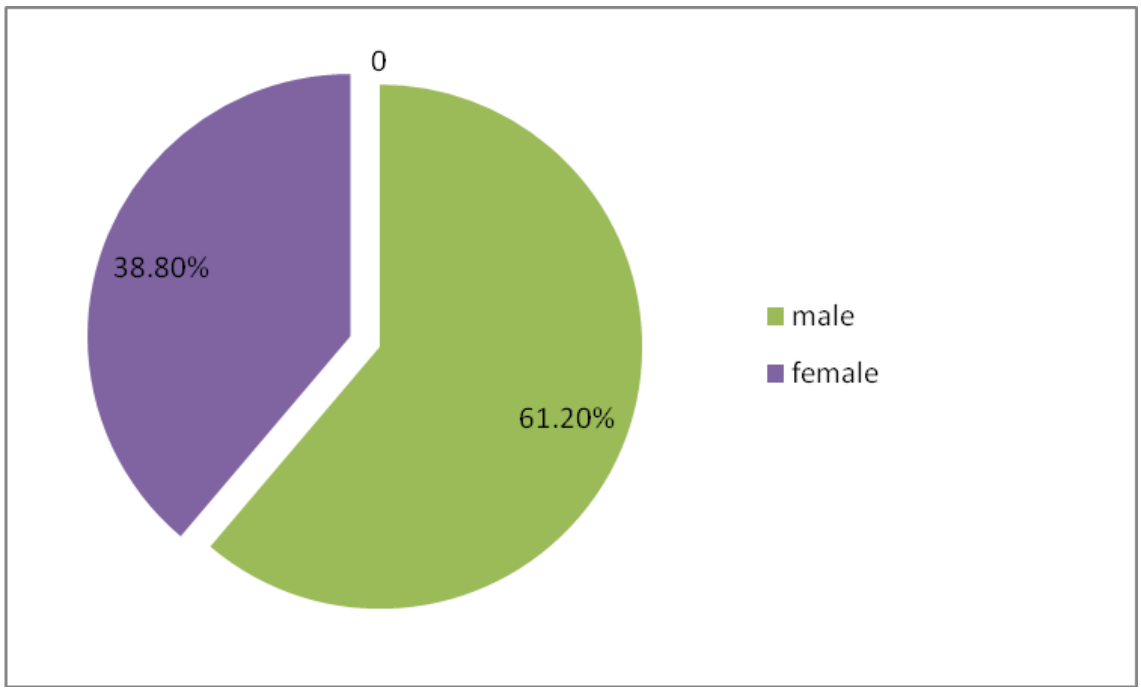
Illustration no.1: **Age distribution of Haemophilus influenzae infections**



Gender distribution:

Among the isolated strains of Haemophilus influenzae 63 (61.2%) were in male and 40(38.8%) were in female patients. (Illustration no 2)

Illustration no.2: **Gender distribution of Haemophilus influenzae infections**

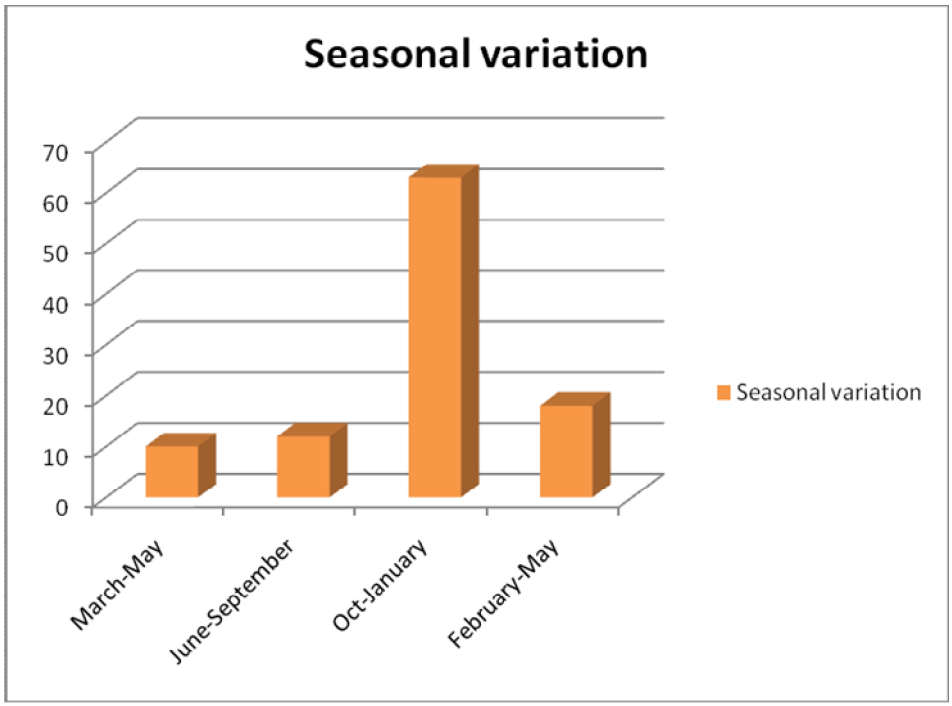


Seasonal variation:

In the winter months of October to January, 63 (61.1%) strains of Haemophilus influenzae were isolated. In summer months of March-May, 10(9.7%) isolates were identified. In the months of June to September, 12(11.65%) isolates were identified and 18 (17.47%) isolates from February to May of 2015.

(Illustration no 3)

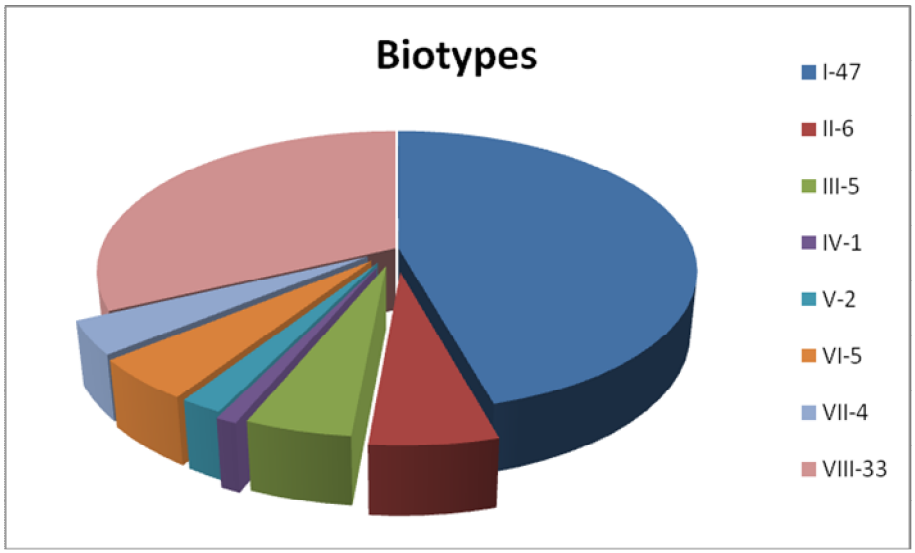
Illustration no.3: **Seasonal variation of Haemophilus influenzae infections**



Biotypes:

Haemophilus influenzae can be divided in to eight biotypes based on three biochemical tests Indole, Ornithine and Urease production. Out of 103 isolates, strains belonging to all biotypes were detected. Out of them 41.7% belong to biotype I, 7.8% to type II, 4.8% to type III, 1% to type IV, 1.9% to type V, 2.9% to type VI, 3.9% to type VII and 33% to type VIII. (Illustration no 4)

Illustration no.4: **Biotype distribution among Haemophilus influenzae infections**



Biotype /serotype distribution:

Among the 8 different biotypes identified 18 isolates of type b falls under biotype I, 9 isolates under biotype VIII. (Table no.1)

Table no.1: **Biotype distribution among various serotypes of H.influenzae isolates**

Biotypes	Type b	Type a	Type f	Nontype b
I	18	5	5	19
II	2	1	1	2
III	2	1	0	2
IV	1	0	0	0
V	2	0	0	0
VI	3	0	0	2
VII	1	1	0	2
VIII	9	5	4	15

Serotype:

All the isolates of Haemophilus influenzae were serotyped with type b,type a and type f antisera.A total of 34(33%) were type b and the remaining 69 were non-type b Among the 69 non type b strains, 46.6% were other serotypes, 10.7% were type a and 9.7% were type f. (**Table no.2a**)

Table no.2a: **Serotype distribution of Haemophilus influenzae infections**

Serotypes	Frequency	Percent
Type b	34	33
Type a	11	10.7
Type f	10	9.7
Other Nontype b	48	46.6
Total	103	100.0

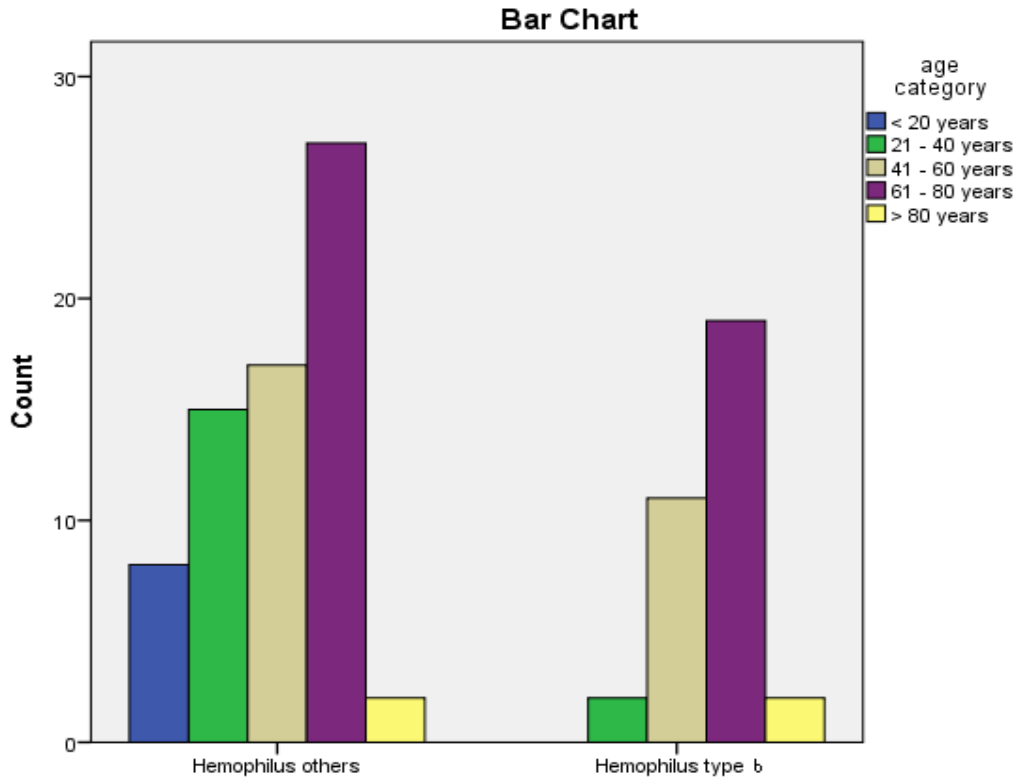
Serotypes in different age groups:

Among the age wise distribution noted, 27 of Haemophilus influenzae were isolated as Nontype b in 60 -80 years and 19 of type b from the same age group. Others are shown in the following table. (**Table no. 2b**)

Table no. 2b: **Serotype distribution of H.influenzae in different age groups**

Serotypes	age category					Total
	< 20 years	21 - 40 years	41 - 60 years	61 - 80 years	> 80 years	
H.influenzae nontypeb	8	15	17	27	2	69
H.influenzae type b	**P value (0.043)** 0	2	11	19	2	34
Total	8	17	28	46	4	103

Illustration no.5: **Serotype distribution of H.influenzae in different age groups**



Comparison of Haemophilus influenzae serotype b in different age groups showed that no cases were identified in age groups less than 20 years after the introduction of vaccine in our tertiary care hospital. The statistical analysis of Chi-square tests gives a P value of 0.043 and since this value is less than 0.05, makes it as a significant study.

Antibiotic susceptibility of Haemophilus influenzae:

Antibiotic sensitivity testing of 103 isolates of Haemophilus influenzae showed that 91.2% were sensitive to Ceftrioxzone, 96.1% to Azithromycin, 94.2% to ciprofloxacin, 85.4% to Ampicillin, 97.08% to Tetracycline and the 65.04% Cotrimoxazole shown in Table No.3

Table no.3: **Antibiotic susceptibility of Haemophilus influenzae by**

Kirby bauer disc diffusion method

Antibiotics	Sensitivity		Resistance	
	Number	percentage	Number	percentage
Ceftriaxone	94	91.2%	9	8.73%
Azithromycin	99	96.1%	4	3.90%
ciprofloxacin	97	94.2%	6	5.90%
Ampicillin	88	85.4%	15	14.6%
Tetracycline	100	97.08%	3	2.91%
cotrimoxazole	67	65.04%	36	35.00%

Detection of Ampicillin resistance:

- Detection of Ampicillin resistance by Kirby-Bauer disc diffusion method:

Among the 103 isolates of Haemophilus influenzae isolated, 15(14.6%) strains showed Ampicillin resistance. (Table no 4)

Table no.4: **Ampicillin resistance among H.influenzae isolates**

H.influenzae	Frequency	Percent
Ampicillin sensitive	88	85.4
Ampicillin resistant	15	14.6
Total	103	100

- Detection of Ampicillin resistance by the phenotypic acidimetric method:

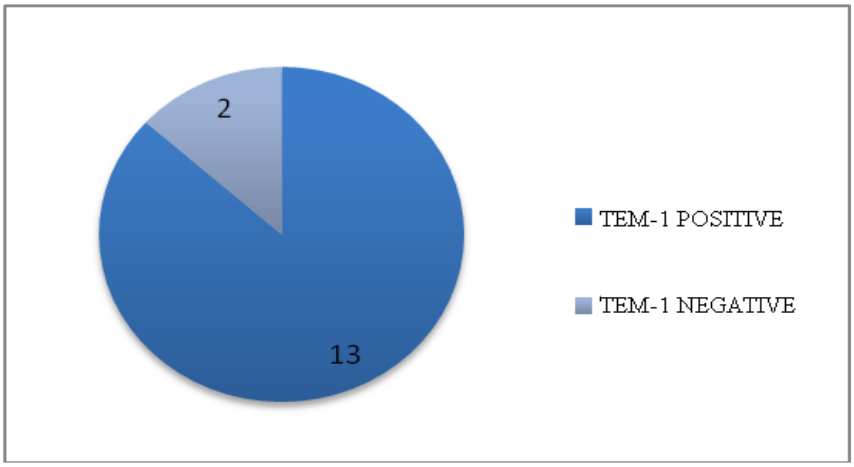
All the 15 (14.6%) isolates of Ampicillin resistant Haemophilus influenzae showed beta lactamase production.

- Detection of Ampicillin resistance by the genotypic method- PCR

Out of the 15 resistant strains of Haemophilus influenzae which showed positive in phenotypic acidimetric method were then subjected to detection of the plasmid mediated resistance gene TEM-1. Among them, 13(12.6%) showed positive for TEM-1 gene. (Illustration no.5)

Illustration no.6: **Molecular Characterization of Ampicillin resistant isolates**

(n= 15)



Association with other diseases:

Among the 103 isolates of Haemophilus influenzae isolated, (3) 2.9% of cases were associated with Pneumococcal pneumoniae and (4) 3.88% of cases were with Klebsiella pneumoniae. (Table. no 5)

Table .no 5 **Association of H.influenzae with other respiratory tract infections**

Diseases	
Tuberculosis	0
Pneumococcal pneumoniae	3
Klebsiella pneumoniae	4

Lower respiratory tract infections from which H.influenzae were isolated:

Out of 103 isolates, 84 were from inpatient samples and 19 were from outpatient samples. In the 84 inpatient cases, 33% of them presented with history of respiratory tract infections and were diagnosed as the cases of pyrexia of unknown origin, 26.2% associated with history of Bronchial asthma,18.44% associated with COPD, 2.9% associated with sinusitis and 0.9% associated with Otitis media. (Table no.6)

Table no.6: **Lower respiratory tract infections from which H.influenzae were isolated among inpatients**

(n=84)

PUO	34
COPD	27
Bronchial asthma	19
Sinusitis	3
Otitis media	1
Meningitis	0

Figure – 1 Gram stain showing pleomorphic Gram negative bacilli

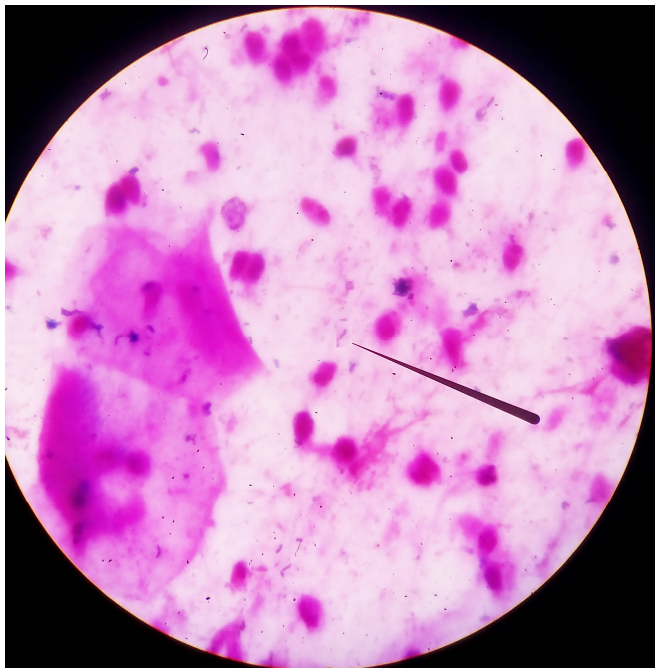


Figure – 2 Blood agar showing Satellitism

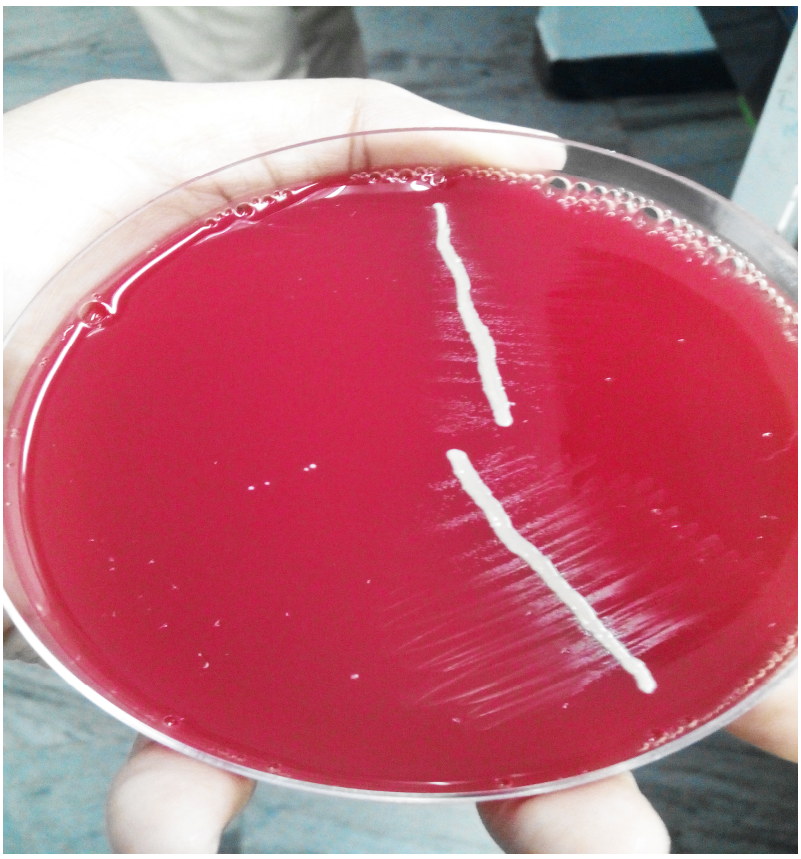


Figure – 3 Chocolate agar showing tiny colonies of H.influenzae

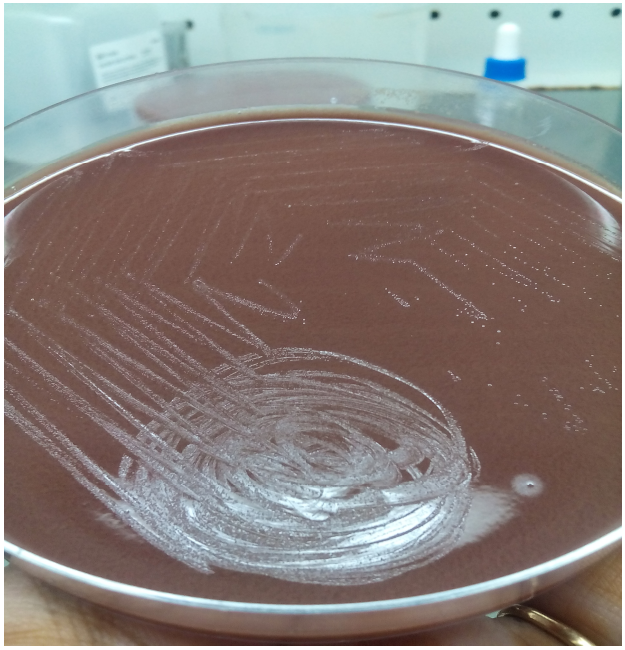


Figure – 4 FILDES agar showing tiny colonies of H.influenzae

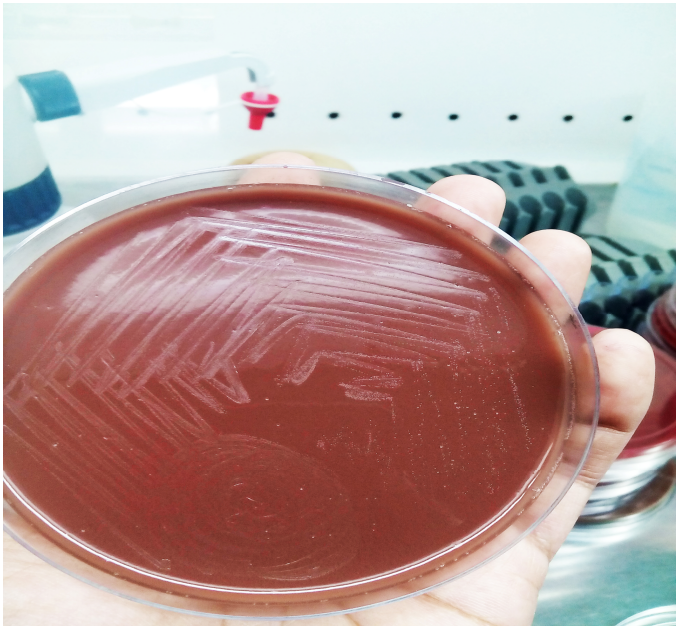


Figure – 5 H.influenzae showing XV requirement on Muller Hinton Agar

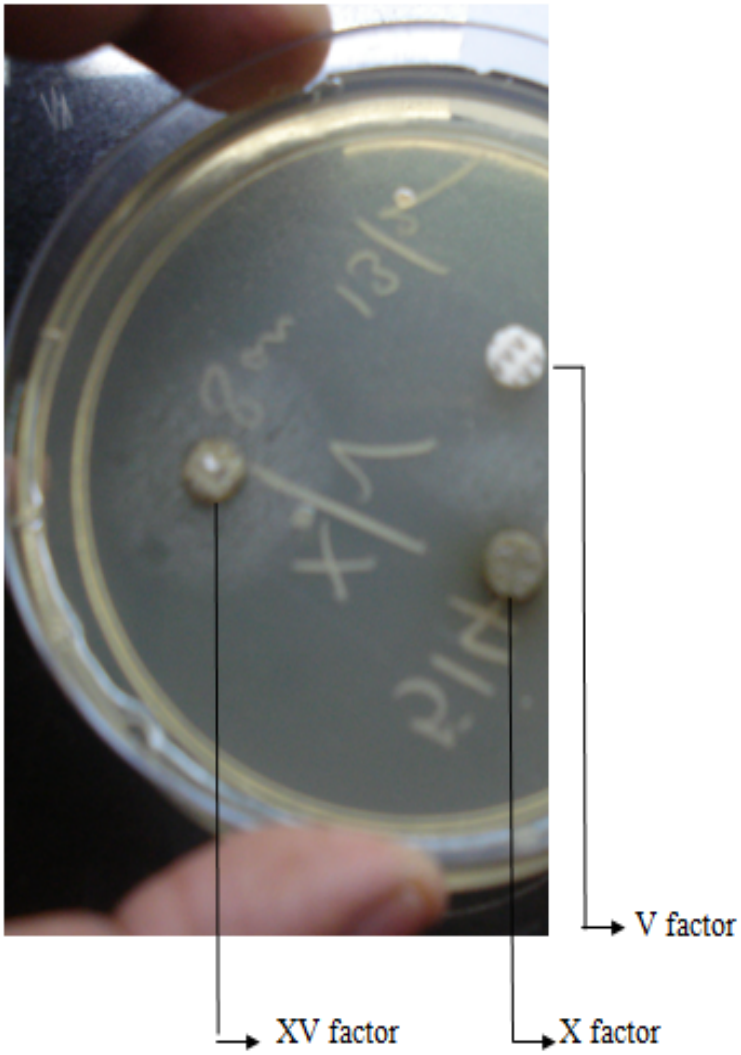
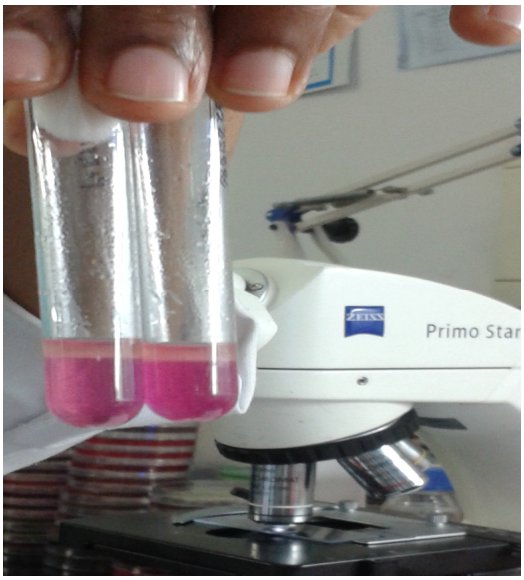


FIGURE- 6-Porphyrin synthesis test

Positive test-pink colour in water phase



Negativetest-nocolour

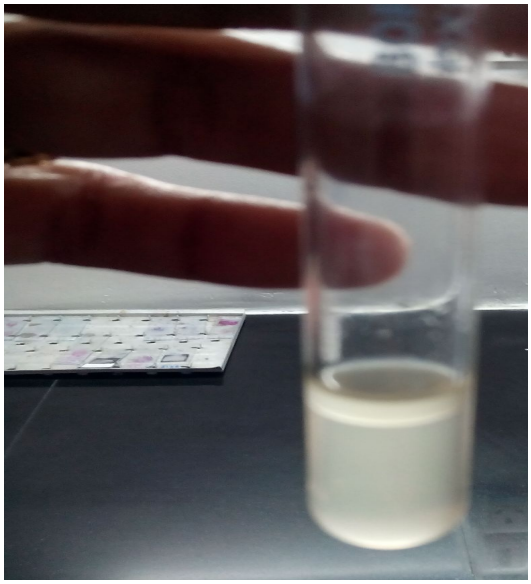


Figure – 7 Biotyping of Haemophilus influenzae

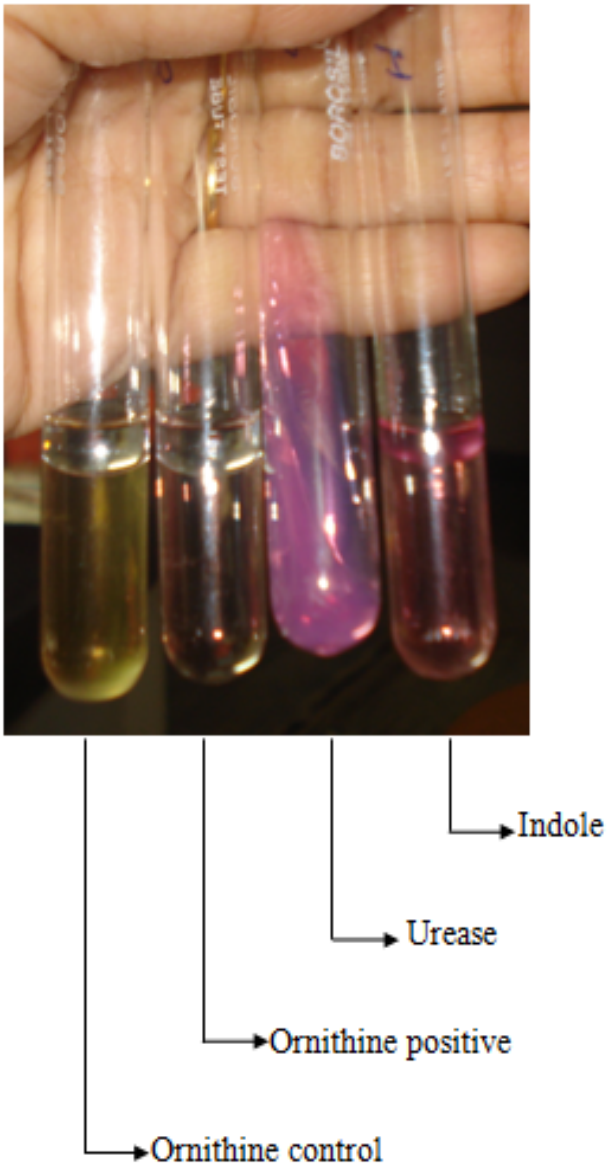


Figure – 8 Seroagglutination with type b, a and f antiserum of H.influenzae

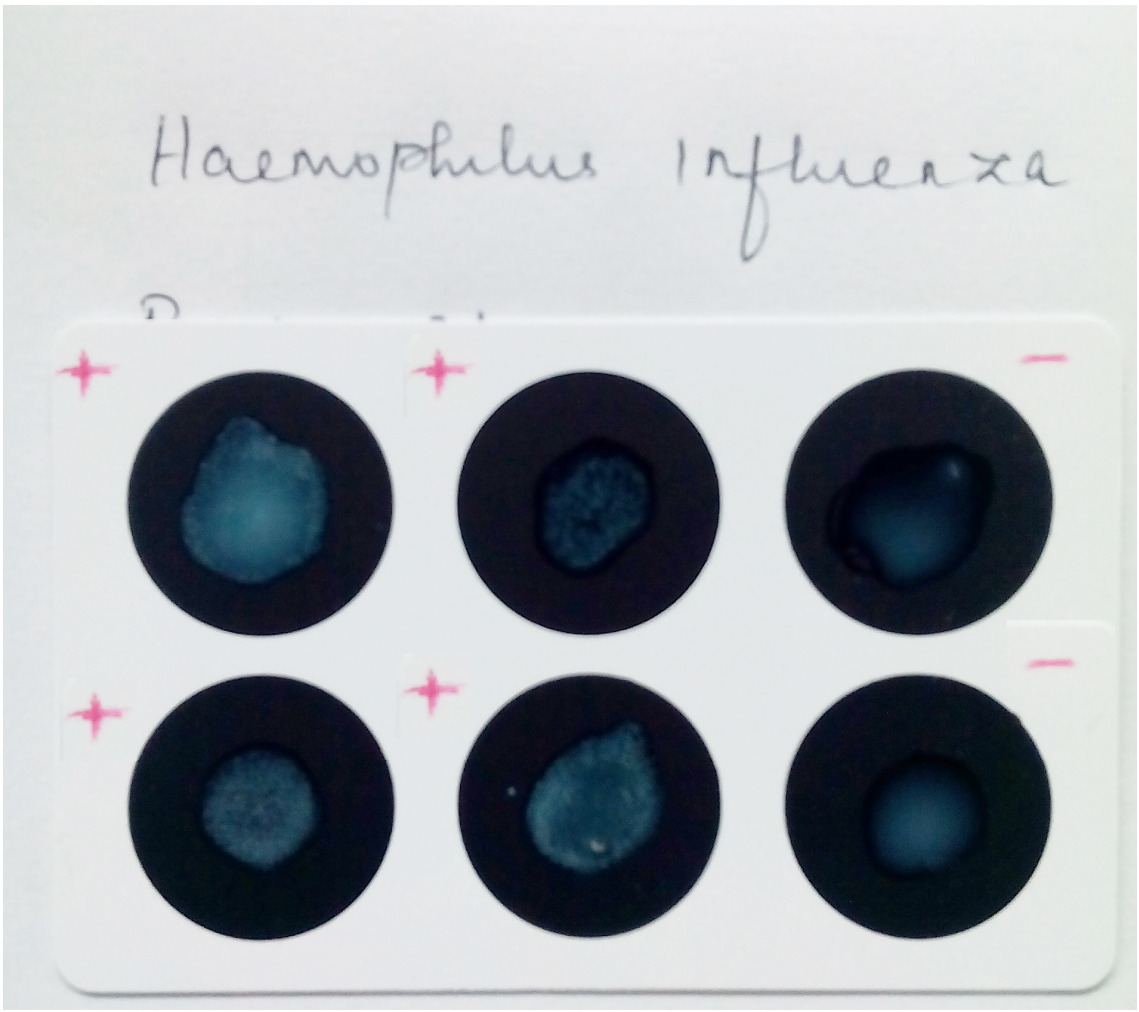
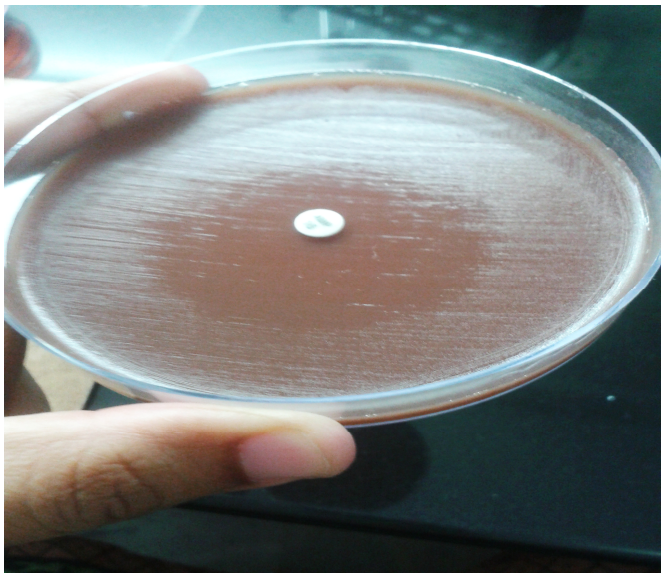
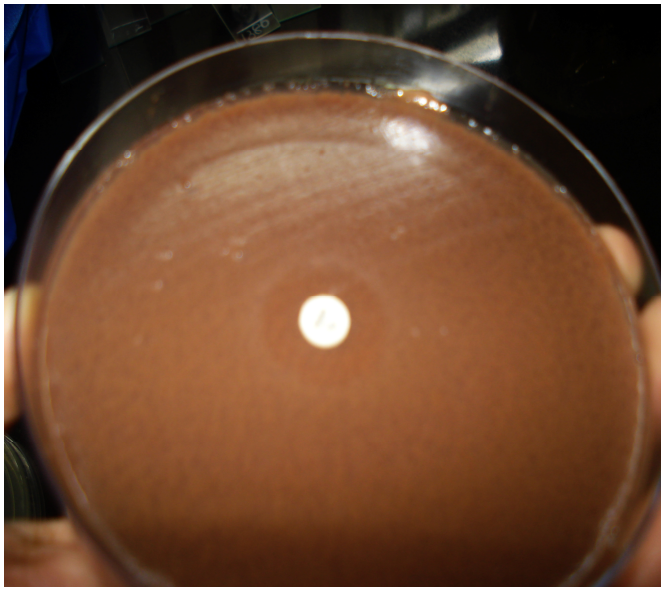


Figure – 9 Ampicillin sensitive and resistant strains

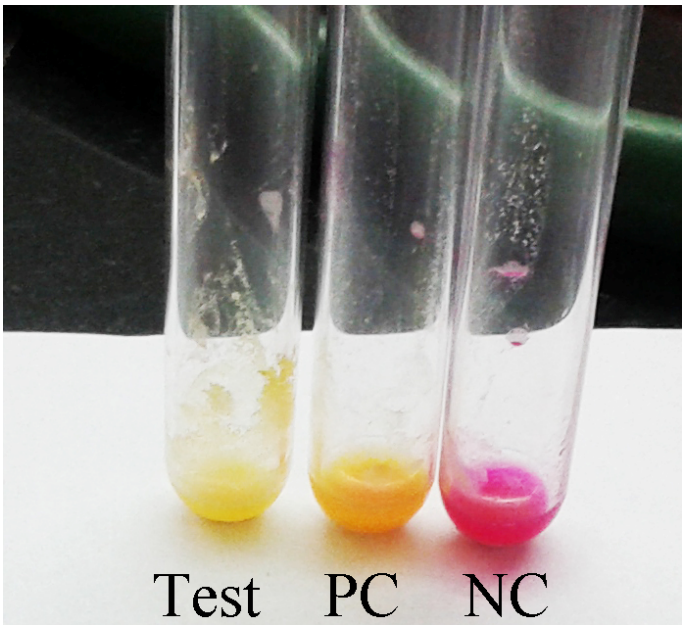


Ampicillin sensitive (Zone size = 30mm)



Ampicillin resistant (Zone size=17mm)

Figure – 10 Phenotypic – Acidimetric method



PC-(POSITIVE CONTROL)-Yellow

NC-(NEGATIVECONTROL)-Pink

Figure -11 Molecular method PCR – TEM – 1 (526 bp)

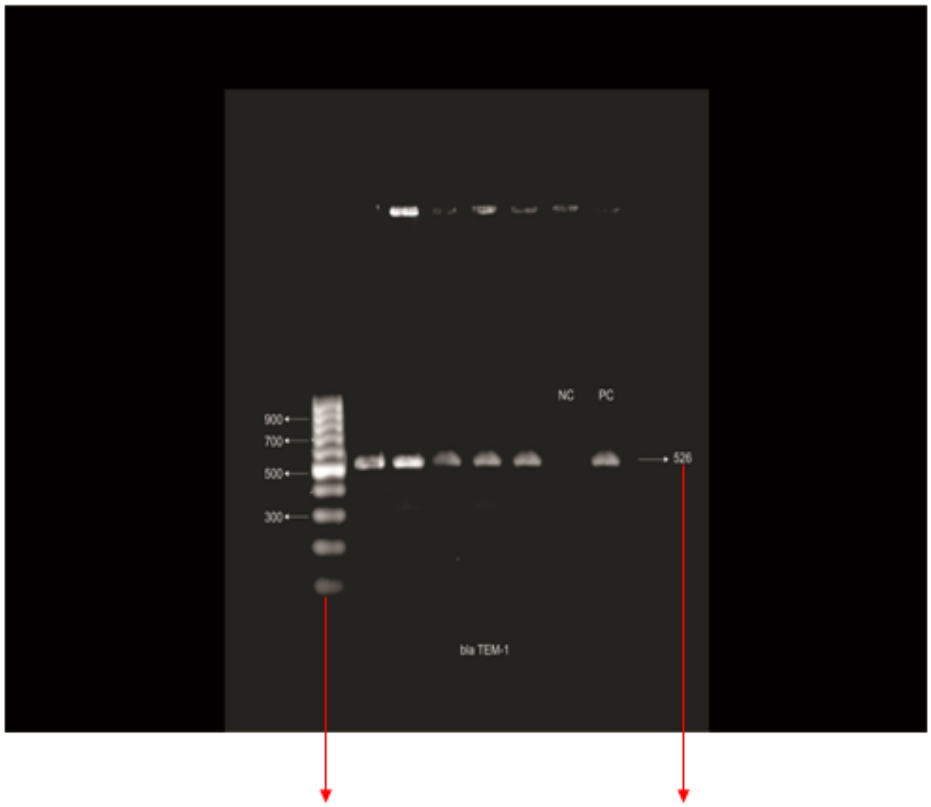
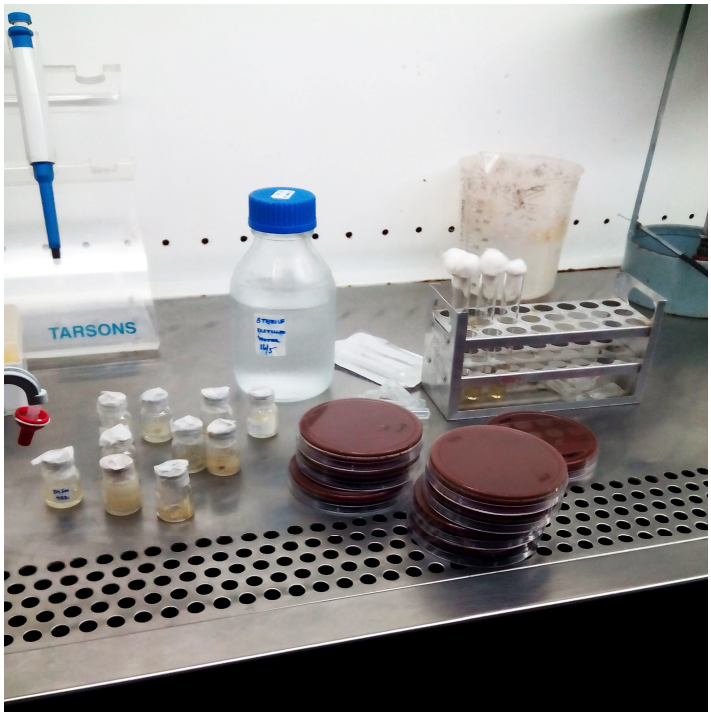


Figure-13 Preservation and storage by Lyophilisation



Discussion

Discussion

Haemophilus influenzae causes a variety of community acquired respiratory infections, including sinusitis, acute otitis media, bronchitis and pneumonia. In olden days, these infections were mainly caused by Haemophilus influenzae serotype b (Hib).

In this study, majority of the cases (33%) presented with history of respiratory tract symptoms, 18% were associated with COPD, 2% associated with sinusitis and 0.9% associated with Otitis media. In the Urwin et al study, 63% of cases presented with history of respiratory infections and 33% presented with COPD[69].

In this study out of 103 isolates, 95 % were seen in adults, where as others report a range of 72-74% in their studies done during the period of 1996-2004[89, 69, 96]. Still earlier studies done during the period from 1993-1997, show a high rate (86%) of isolation of Hib amongst children below 5 years of age[90]. A Costa Rican study have reported a significant decline in the incidence of type b H.influenzae when the data from 1992 -1997 was compared with that between 1999 and 2004 [91]. By June 2011, Hib vaccine was included in the National Immunization program of 170 countries in all regions of the world. After that the incidence of Hib invasive disease and oropharyngeal carriage in young children has drastically decreased wherever vaccination programs have been implemented [89, 96, 97, 98].

The H.influenzae isolates in this study were more commonly seen among male patients (61%), which is similar to other studies[9,69]. In contrast, a study reports higher prevalence among female elderly patients, whereas a lower prevalence in female children[2].

In this study the isolation of H.influenzae was more (61%) during the winter months which are similar to other studies [6,103]. In another study, an increase in the frequency of isolation of H.influenzae (68%) isolates was noted in rainy season when compared to that (32%) in the dry season[91, 99, 103]. Serotyping of the isolates showed that the isolation of H influenzae type b has become very meager in many studies, ranging from 1-3%, but in our study 33% belonged to this serotype[102].

Table no.1: **Comparison of H.influenzae isolation in this study with other studies**

Study name/period	Type b	Non type b	Type f	Type a	Type e
This study	33%	67%	10%	11%	-
Jessica R. etal[101] (1989)	80%	17%	-	-	-
Amita Jain etal[68] (2006)	31%	68%	-	-	-
Jessica R. etal[101] (2008)	3%	97%	18%	2%	6%
Puig, Carmen [102](2008-2013)	1%	99%	13%	-	

Haemophilus influenzae are divided in to eight biotypes based on the three biochemical tests, namely, Indole, Ornithine and Urease production. In my study, Out of the 103 Haemophilus influenzae isolates, strains belonging to all biotypes were detected. Out of them most of the isolates (44%) belonged to biotype I. There is no concurrence among the various studies on the biotype isolated, where in the predominant biotype varies.

Table no.2: **Comparison of biotype distribution of H.influenzae with other studies**

Study name	Predominant biotype	Percentage	Year
Our study	I	44%	2013
Amita etal[68]	III	25%	2006
Landgraf etal[92]	I	70.9%	1993
J.Wallace etal[100]	IV	38%	1983

In this study, among the 103 isolates of Haemophilus influenzae, majority of them showed sensitivity to Tetracycline (97%), Azithromycin (96%), Ciprofloxacin (94%), Ceftrioxzone (91%) followed by Ampicillin (85%) and Cotrimoxazole (65%).The sensitivity to tetracycline had increased from 54% in the year 2000 to 94.5% in 2012 which is similar to this study.[103]

Haemophilus influenza infections showed a good response to usage of third generation cephalosporin especially Ceftrioxzone (91%) and it correlates well with studies done from 2005 to 2007 in the United States.[103, 105] In developed countries, the resistance to cephalosporins is very low and it varies from 0.1% to 0.3% percent which correlates with this study as well.[111]

Haemophilus influenzae non type b is an emerging pathogen commonly causing respiratory infections in adults particularly sinusitis, otitis media. These are now frequently treated with fluoroquinolones and their sensitivity has increased to 99% in studies done from 2000-2013[106]. Our study also showed a 94% susceptibility to Ciprofloxacin. A global study (SENTRY) performed by American and European institutions found that 0.15% of H. influenzae isolates were resistant to Fluoroquinolones [107].

Generally, Gram positive bacteria are more susceptible to macrolides, but H influenzae among many of the Gram negative bacteria also show a high susceptibility to macrolides like Azithromycin. In our study 99% were susceptible to Azithromycin, which was similar to other studies.[108] High level resistance to macrolides in H.influenzae (0.5%-2%) is rare as is reported in many studies. [108,109,110]

There was no difference in susceptibility between type b and non-type b isolates to Ceftrioxzone, tetracycline,azithromycin and Ciprofloxacin except with Ampicillin and Cotrimoxazole in which type b isolates shows higher resistance(26% and 47%) when compared to nontype b isolates .[111,103]

In my study out of the 103 isolates of Haemophilus influenzae, 35% of H.influenzae isolates showed resistance to Cotrimoxazole which is similar to the study done by Maestro etal in rural (13%) and urban children (46%) suffering from ARI infections [112] and in V.L.Nag etal study reporting 33% resistance. [111]

Beta lactamase mediated resistance is seen in 15% isolates in this study, whereas in multicentre studies done in US between 1984-1999 it was 15% to 36% [111] and in recent studies done in China, the percentage of Ampicillin sensitive strains were progressively decreased from 96% in the year 2000 to 61% in 2012.[103] The resistance detected was higher among type b (60%)strains when compared to non type b strains(40%) which is similar to study by V.L.Nag et al (30% and 20%) suggesting the strains that are invasive shows high Ampicillin resistance.[111]

All the resistant isolates (15) produced beta-lactamase enzyme when screened by the phenotypic acidimetric method, but when analyzed by molecular method, only 13 isolates (86%) were positive for TEM-1 gene. Beta lactamase production may also be due to presence of ROB-1 gene. Whereas in another study 11% showed Ampicillin resistance, in which 73% were beta-lactamase positive and carried TEM-1 gene and none were positive for ROB-1 gene [104]. Many studies have shown that the isolation of TEM-1 gene (93%) is more when compared to the ROB-1gene isolated (5%)[83, 95].

Summary & Conclusion

Summary & Conclusion

- During the study period from April 2014 - August 2015, 1540 purulent respiratory samples from patients with suspected pneumonia were processed.
- Out of 1540 samples, Haemophilus influenzae was isolated in 103 samples.
- Majority of the isolates (49%) were from the elderly, of more than 60 years of age.
- Incidence of Haemophilus influenzae was higher in males (60%).
- The isolation of Haemophilus influenza was more (61%) during the winter months of October to January.
- Majority of Haemophilus influenzae belonged to biotype I (42%)
- Among the isolates, type b serotype (33%) was less when compared to non type b serotypes (67%).
- Type b serotype isolates were more among the age group of 60-80 years and none was seen in less than 5 years of age.
- Haemophilus influenza showed high sensitivity to Tetracycline (97%), Azithromycin (96%), ciprofloxacin (94%), Ceftrioxzone (91%) when compared to Ampicillin (85%) and the Cotrimoxazole (65%).
- There is no difference in the susceptibility to drugs between type b and Non type b isolates.
- Beta lactamase H.influenzae (15%) was detected by the phenotypic acidimetric method. Out of them, 86% showed TEM-1 beta lactamase gene by molecular method PCR.

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Appendix

Appendix:

Blood agar:

- Sterile defibrinated sheep blood 7ml
- Nutrient agar (melted) 100ml

Add sterile defibrinated sheep blood to the melted nutrient agar at 45 to 50 ^{0} C.Pour 20 ml in to each Petri dish about 4mm depth.

Chocolate agar (CA):

CA is heated blood agar.

- Sterile defibrinated blood 7ml
- Nutrient agar 100ml

Melt the nutrient agar .When the temperature is about 45-50 ^{o} C add the blood and mix well. After adding the blood, heat it in a water bath slowly bringing up the temperature to 75^{o} c with constant agitation. Special care should be taken to avoid fluctuation in the temperature.

CHOC-VBC:

Chocolate agar is prepared as such above and the following antibiotics are added in the concentrations of:

- Vancomycin…..5 microgram/ml
- Bacitracin……300 microgram/ml
- Clindamycin….1 microgram/ml

Fildes agar:

We have to prepare BHI chocolate agar and to that 5% Fildes enrichment broth (commercially available from HIMEDIA) is added on to it.

- BHI agar….3.75 gm
- Distilled water …100ml

Autoclave after adjusting pH to 7.4

- Fildes enrichment broth…5ml
- Distilled water… 100ml

Autoclave after adjusting pH to 7.4

Cool the media to 45 to 50^0 c.

Add 5ml of sheep blood and then heat the media to 80-85^0c and make it to chocolate agar. Then add 5ml of Fildes enrichment broth to it and allow the media to set.

Biotyping:

Indole test:

Reagent:

- P-Dimethylaminmobenzaldehyde 10g
- Amyl or isoamylalcohol 150ml
- Concentrated HCL 50ml

Medium:

- Peptone 20g
- Sodium chloride 5g
- Distilled water 1litre

Adjust the Ph to 7.4.Dispense and sterilize by autoclaving at 121 ^{0}c at 15 minutes.

Method:

Inoculate the bacterial colonies in to the above medium aseptically and incubate at 37 ^{0} c for 18-24 hours. Next day add the kovac's reagent approximately 0.5ml (5 drops) along the sides of the tube.

INFERENCE:

The pink color seen in the water layer is considered positive. Sometimes a period of 96 hours is required for optimum accumulation of indole.

- Positive control: E.coli
- Negative control: Klebsiella

Urease test:

Principle:

To determine the ability of the organism to split urea in to two molecules of ammonia by the action of the enzyme urease with resulting alkalinity.

Medium:

Christensen's medium:

- Peptone 1g
- Sodium chloride 5g
- Monopotassium phosphate 2g
- Glucose 1g
- Urea 20g
- Phenol red 0.012g
- Agar 15g
- Distill water 1litre

pH indicator : phenol red .

Maintain pH 6.8.Then autoclave at 121 ^{0} c at 15 minutes and then allow it to cool at 45-50 ^{0} c.Pour the media with a slant and butt.

Method:

Inoculate heavy inoculum in to the medium and incubate at 37 ^{o} c for 18-24 hours. If the organism utilizes the urea, ammonia is formed and the medium results in alkalinity.

Inference:

Appearance of pink colour indicates the presence of urease enzyme

- Positive control: Proteus spp
- Negative control: E.coli

Ornithine [6]:

Principle:

To measure the enzymatic ability of an organism to decarboxylate an amino acid to form an amine with resulting alkalinity.

Medium:

Moller's decarboxylase media:

- Peptone 5g
- Beef extract 5g
- Bromocresol purple 0.1g
- Cresol red 0.005g
- Pyridoxal 5mg
- Glucose 0.5g
- l-ornithine dihydrochloride 10g
- Distilled water 1litre.

Maintain Ph. 6 .

Autoclave at 121 ^{0} c at 15 minutes.

Cool at 45-50 ^{0} c

Method:

Inoculate light inoculum in to the medium and incubate at 37 ^{o} c for 18-24 hours. A control tube without an amino acid should be inoculated .Overlay the test and the control tubes with sterile paraffin oil .Under these conditions the oxygen in the medium is used up by the organism and this will control the pH.

Inference:

Purple color change in the medium indicates the decarboxylation of ornithine.

- Positive control: Enterobacter cloacae
- Negative control: E.coli

Porphyrin synthesis test:

This test indicates the absence of requirement of X factor. If &-Amino levulinic acid is provided to the bacterium, it synthesizes it and excretes porphobilinogen and other porphyrins. This test is carried out by using kovac's indole reagent (para dimethyl amino benzaldehyde) and formation of pinkish red colur in the lower water phase indicates the synthesis of porphyrin and absence of X factor requirement.

- Positive control: Staphlococcus aureus [87]
- Negative control:Haemophilus influenzae ATCC 49247

Substrate used:

- &ALA -2mmol/lit
- $Mgso_4$ - 0.8 mmol/lit
- Sodium phosphate buffer 0.1mmol/lit

pH 6.9

- Distribute 0.5 ml volumes of the substrate in small tubes

Add large loopful of bacteria from the plate

- Incubate for 4 hours.Add 0.5ml of Kovac's indole reagent (para dimethyl amino benzaldehyde)
- Vortex and allow the phases to separate.
- Reddish pink colour in the lower water phase indicates the synthesis of porphyrin and absence of X factor requirement.

Annexure

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

April 10, 2014

To
Dr D Sai Keerthana
Postgraduate
Department of Microbiology
PSG IMS & R
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore -4, has reviewed your proposal on April 4, 2014 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your study proposal entitled:

"Isolation, characterisation, antibiotic susceptibility pattern of hemophilus influenzae isolated from respiratory samples"

The following documents were received for review:

1. Duly filled application form
2. Proposal
3. Confidentiality statement
4. Application for waiver of consent
5. CV
6. Budget

After due consideration, the Committee has decided to approve the study.

The members who attended the meeting at which your study proposal was discussed are as follows:

Name	Qualification	Responsibility in IHEC	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
Dr P Sathyan	DO, DNB	Clinician, Chairperson	Male	No	Yes
Dr S Bhuvaneshwari	M.D	Clinical Pharmacologist Member - Secretary	Female	Yes	Yes
Dr Sudha Ramalingam	M.D	Epidemiologist Alt. Member - Secretary	Female	Yes	Yes
Dr Y S Sivan	Ph D	Member –Social Scientist	Male	Yes	Yes

The approval is valid for one year.

We request you to intimate the date of initiation of the study to IHEC, PSG IMS&R and also, after completion of the project, please submit completion report to IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

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This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Yours truly,

10.4.14

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr S Bhuvaneshwari
Member - Secretary
Institutional Human Ethics Committee

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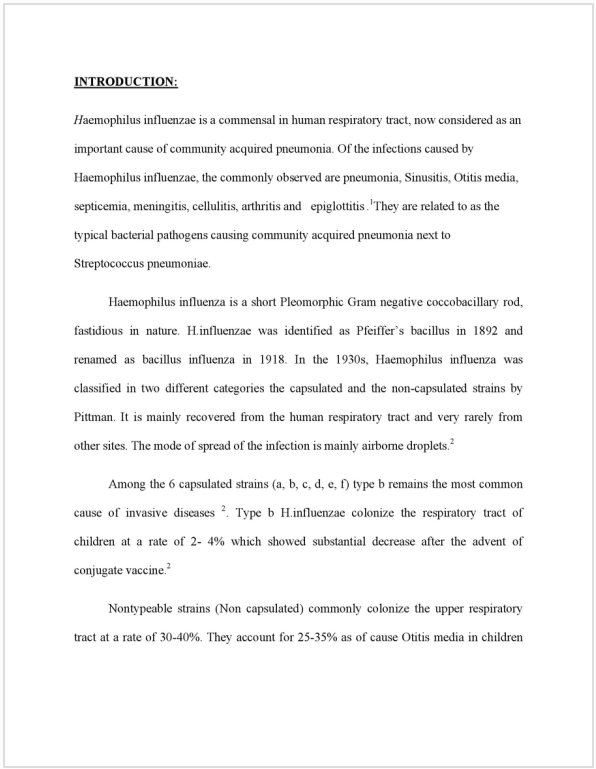

INTRODUCTION:

*H*aemophilus influenzae is a commensal in human respiratory tract, now considered as an important cause of community acquired pneumonia. Of the infections caused by Haemophilus influenzae, the commonly observed are pneumonia, Sinusitis, Otitis media, septicemia, meningitis, cellulitis, arthritis and epiglottitis .[1]They are related to as the typical bacterial pathogens causing community acquired pneumonia next to Streptococcus pneumoniae.

Haemophilus influenza is a short Pleomorphic Gram negative coccobacillary rod, fastidious in nature. H.influenzae was identified as Pfeiffer's bacillus in 1892 and renamed as bacillus influenza in 1918. In the 1930s, Haemophilus influenza was classified in two different categories the capsulated and the non-capsulated strains by Pittman. It is mainly recovered from the human respiratory tract and very rarely from other sites. The mode of spread of the infection is mainly airborne droplets.[2]

Among the 6 capsulated strains (a, b, c, d, e, f) type b remains the most common cause of invasive diseases [2]. Type b H.influenzae colonize the respiratory tract of children at a rate of 2- 4% which showed substantial decrease after the advent of conjugate vaccine.[2]

Nontypeable strains (Non capsulated) commonly colonize the upper respiratory tract at a rate of 30-40%. They account for 25-35% as of cause Otitis media in children

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Abstract

Introduction:

Haemophilus influenza is strictly a human pathogen responsible for many diseases like community acquired pneumonia, meningitis, sinusitis, epiglottitis and otitis media. Haemophilus type b is the most common strain responsible for all the infections. Non type able strains are at emerging risk. Ampicillin resistance is being reported and is due to production of betalactamases. Ampicillin resistance is being reported and is due to production of betalactamase, but rarely can also be due to aminoacid substitutions in the Penicillin binding proteins (PBP) which is known as BLNAR

AIM:

To isolate and characterize Haemophilus influenzae as pathogen from purulent respiratory samples over a period of 15 months.

Materials and Methods:

All purulent respiratory samples were processed for isolation of Haemophilus influenzae. They were identified as H. influenzae based on its X&V factor requirement and Porphyrin synthesis test. Serotyping of the isolates was done using 'b', 'a' and 'f' typing sera. Antibiotic susceptibility testing was done with reference to detection of Ampicillin resistance. Ampicillin resistance was characterized by phenotypic and genotypic methods. PCR was done for detection of TEM -1 which codes for betalactamases enzyme.

Results:

Out of 103 Haemophilus influenzae isolates, majority of them were isolated from elderly male patients (49%) and show higher incidence during the winter months .Due to the vaccination available against type b Haemophilus influenzae, Non type b Haemophilus influenzae shows higher risk (67%) when compared to type b (33%). None of the cases of type b is seen in children below 5 years of age. Haemophilus influenzae shows higher sensitivity pattern to ceftriaxone (91%),Azithromycin (96%)and Tetracycline (97%). There is no difference in the susceptibility to drugs between type b and Non type b isolates. Beta lactamase positive H. influenzae were seen in 15 isolates .Out of them, 13 showed TEM-1 beta lactamase gene by PCR.

KEYWORDS: satellitism, XV factor requirement, Porphyrin synthesis test, Fildes agar,TEM-1